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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/543, C12N 15/10

A1

(11) International Publication Number: WO 99/15897

(43) International Publication Date: 1 April 1999 (01.04.99)

(21) International Application Number: PCT/US98/19425

(22) International Filing Date: 17 September 1998 (17.09.98)

(30) Priority Data:
60/059,508 19 September 1997 (19.09.97) US

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(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

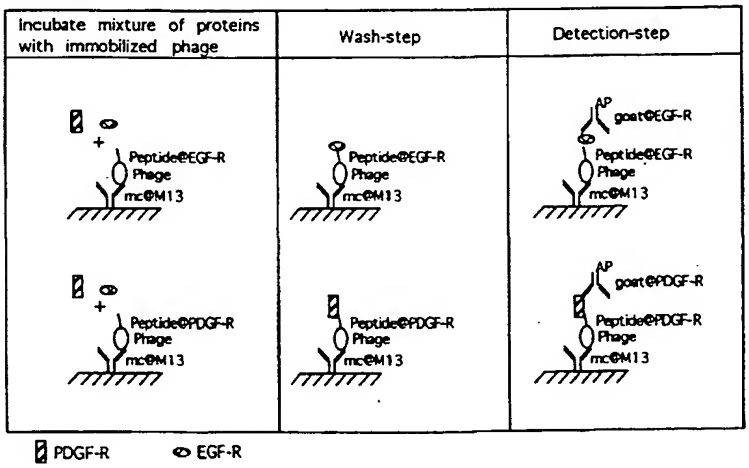
Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SUBTRACTIVE PROTEIN SCREENING FOR GENE IDENTIFICATION

ELISA-type experiment using specific immobilized peptide-phages to capture specific target protein receptors.



(57) Abstract

A method of using antibody expression clones (e.g. bacteria, bactenophage expressing single chain antibodies) bound to a solid support for identifying genes which are differentially expressed between two cell types is described. The invention can be used to identify proteins and genes which are differentially expressed between any two cells, for example diseased and non-diseased cells, cells exposed to an environmental factor or an exogenous substance and those not exposed, cells of different organs or organisms, or cells at different stages of a developing organism. Antibody expression clones can also be used to identify intracellular proteins which are involved in cellular responses.

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SUBTRACTIVE PROTEIN SCREENING FOR GENE IDENTIFICATION

FIELD OF THE INVENTION

The invention relates to the area of gene expression. More particularly, the invention relates to the area of identifying genes which are differentially expressed between two cells.

BACKGROUND OF THE INVENTION

Phenotypic differences between tissues are attributable to differential gene expression. For example, neoplastic or other diseased cells express genes which are not expressed by non-diseased cells, and these expressed genes contribute to the diseased phenotype. Identification of such genes would provide the art with tools to manipulate their expression so as to control a particular disease state.

Current methods for identifying differentially expressed genes relies on RNA substraction or differential display of mRNA (Lee *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:2825-2829). Substraction methods are sensitive and detect somewhat rare mRNAs. However, the method recovers genes incompletely and selects for genes in only one direction at a time during two way comparisons between cells.

Differential display can be used to provide a picture of mRNA composition of cells. A powerful application of differential display is the ability to clone differentially expressed RNAs in differing biological systems. Due drawback of the method is the problem of false-positive differences. Furthermore, there is no distinction between mRNA molecules which encode proteins. Thus, there is a need in the art for methods of identifying differentially expressed proteins.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for identifying an antibody which binds to a differentially expressed protein.

It is another object of the invention to provide a method for identifying a gene which is differentially expressed between two cells.

It is yet another object of the invention to provide a solid support comprising a pattern of antibody expression clones.

It is still another object of the invention to provide a kit for identifying a gene which is differentially expressed between a first cell and a second cell.

It is even another object of the invention to provide a method of identifying a gene which encodes an intracellular protein which participates in a cellular response.

These and other objects of the invention are provided by one or more of the embodiments described below.

One embodiment of the invention provides a method for identifying an antibody which binds to a differentially expressed protein. Labeled proteins from a first cell are contacted with a first solid support under conditions where antibodies specifically bind to proteins. The first solid support comprises a pattern of antibody expression clones, wherein the antibody expression clones express fusion proteins comprising single chain antibodies. The single chain antibodies are immunoreactive with proteins of the first cell. A first pattern of immunoreactivity on the first solid support formed by the binding of labeled proteins from the first cell with the single chain antibodies expressed by the antibody expression clones is determined. Labeled proteins from a second cell are contacted with a second solid support. The second solid support comprises the pattern of antibody expression clones. A second pattern of immunoreactivity on the second solid support formed by the binding of labeled proteins from the second cell with the single chain antibodies expressed by the antibody expression clones is determined. The first pattern of immunoreactivity is compared with the second pattern of immunoreactivity. A difference between the first and second patterns of immunoreactivity identifies an antibody which binds to a protein which is differentially expressed between the first cell and the second cell.

Another embodiment of the invention provides a method for identifying a gene which is differentially expressed between two cells. A single chain antibody which binds to a protein which is differentially expressed between two cells is identified as described above. The identified single chain antibody is isolated and used to screen a population of protein expression clones comprising cDNA obtained

by reverse transcription of mRNA isolated from the first cell. A protein expression clone which binds to the single chain antibody is identified as expressing a cDNA encoding a protein which is differentially expressed between the two cells.

Yet another embodiment of the invention provides a solid support comprising a pattern of antibody expression clones. The antibody expression clones express fusion proteins comprising single chain antibodies.

Still another embodiment of the invention provides a kit for identifying a gene which is differentially expressed between a first cell and a second cell. The kit comprises a first solid support comprising a pattern of antibody expression clones. The antibody expression clones express fusion proteins comprising single chain antibodies, wherein the single chain antibodies are immunoreactive with proteins of the first cell. The first solid support can be used to identify a single chain antibody which binds to a protein which is differentially expressed between a first cell and a second cell. The kit also comprises a second solid support comprising a pattern of protein expression clones. The protein expression clones comprise cDNA obtained by reverse transcription of mRNA isolated from the first cell. The protein expression clones express cDNA encoding proteins of the first cell. The second solid support can be screened with the identified single chain antibody to identify a protein expression clone which expresses a cDNA encoding a protein which is differentially expressed between the first cell and the second cell.

Even another embodiment of the invention provides a method of identifying a gene which encodes an intracellular protein which participates in a cellular response. A library of nucleic acid vectors encoding single chain antibodies is introduced into a population of cells. The vectors express single chain antibodies intracellularly, and the single chain antibodies bind to intracellular proteins of the population of cells. The population of cells is contacted with an inducer of a cellular response. The inducer induces the cellular response in a first subset of the population of cells but does not induce the cellular response in a second subset of the population of cells. At least one nucleic acid vector encoding a single chain antibody is isolated from the second subset of the population of cells. The single chain antibody is expressed and used to screen a population of protein expression clones. The population of protein expression clones comprises cDNA obtained by

reverse transcription of mRNA isolated from the population of cells. A protein expression clone which binds to a single chain antibody is identified as expressing a cDNA encoding an intracellular protein which is involved in the cellular response in the population of cells.

The present invention thus provides the art with methods of using clones which express single-chain antibodies to identify both proteins and genes which are differentially expressed between two cells. The two cells can be any two cells, for example diseased and non-diseased cells, cells exposed to an environmental factor or an exogenous substance and those not exposed, cells of different organs or organisms, or cells at different stages of a developing organism.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts an ELISA experiment using specific immobilized peptide-phages to capture specific target protein receptors.

DETAILED DESCRIPTION OF THE INVENTION

Compositions and methods for identifying differentially expressed proteins and genes are provided. The compositions comprise antibody populations which may be bound to a solid support. For purposes of the invention "antibodies" includes polyclonal or monoclonal antibodies, single chain antibodies, intrabodies, antibody fragments including Fab' and $F(ab')_2$ antibody fragments, and the like. While the invention is generally discussed in terms of single chain antibodies it is recognized that the methods of the invention can be practiced with intrabodies, Fab fragments and the like.

It is a discovery of the present invention that antibody expression clones which express single chain antibodies can be used advantageously to identify proteins and genes which are differentially expressed between two cells. Immobilizing the antibody expression clones on a solid support greatly reduces non-specific background problems which plague method using antibody expression clones in solution. Comparing proteins rather than mRNA also produces more reliable data on protein expression, since not all mRNA is translated into protein. Thus, when a differentially expressed protein is identified using the present

invention, it is likely that the identified protein is important to generating differences seen between the compared cells.

Proteins or genes which are differentially expressed between two cells are proteins or genes which are expressed in a first cell and not in a second cell to which the first cell is compared. Differential protein or gene expression can be identified between any two cells. For example, differential protein or gene expression can be identified between a cell which is diseased and a cell which is not diseased. The cell which is not diseased can be any cell which is functionally and morphologically normal, having no obvious genetic, functional, or morphological alterations. The diseased cell can be a neoplastic cell, for example, from a tumor of the breast, colon, stomach, brain, liver, lung, pancreas, reproductive system, or skin. The neoplastic cell can be a cell which has a high probability of metastasizing, such as a melanoma cell (a highly metastatic cell), or one which has a low probability of metastasizing, such as a basal cell carcinoma of the skin (a weakly metastatic cell). The diseased cell can be infected with a pathogen, such as a virus, bacterium, fungus, mycoplasma, protozoan, or prion. A cell which has been exposed to an environmental factor, such as a particular temperature, atmospheric pressure or composition, or gravitational or magnetic field, can be compared with a cell which has not been exposed to the environmental factor. A cell which has been contacted by an exogenous substance, such as a nutrient, toxin, therapeutic agent, or other chemical, can be compared with a cell which has not been contacted with the exogenous substance. It is desirable that the cells compared be as similar as possible, but for the single distinguishing property being studied.

Differential protein or gene expression between cells at different stages of development can also be identified by this method, by comparing a pattern of protein or gene expression in a cell at one developmental stage with a pattern of gene expression in a cell at a different developmental stage. The proteins or genes which are differentially expressed between any of germ cells (oocytes and spermatocytes), embryonic cells (such as cells of a morula, bastula, gastrula, or the neural crest), differentiating fetal cells, juvenile cells (cells at any developmental stage between birth and adulthood), or adult cells can be so identified.

The cells being compared can originate from any tissue of a human, such as brain, liver, skeletal muscle, heart, pancreas, spleen, blood, skin, intestine, stomach, kidney, bladder, thymus, lung, or mammary gland. Freshly isolated cells or cell lines, such as PC12, HCT116, SW480, or HeLa, can be examined for differential protein or gene expression using the present invention. Differential protein or gene expression in cells of other organisms, both vertebrates and invertebrates, can be identified. Similarly, genes or proteins which are differentially expressed in bacterial or yeast cells, for example at different stages of growth, after growth in different media, or after various chemical treatments, can also be identified. Species-specific gene or protein expression can be identified by suing the method of the present invention to compare gene or protein expression in a cell originating from one species with gene or protein expression in a cell originating from a different species. For example, genes or proteins which are expressed in bacterial but not in yeast can be so identified. Genes or proteins which are differentially expressed between two plant cells can also be identified.

The method uses antibodies to identify differentially expressed proteins. Such antibodies can be produced by any method in the art. See, for example, U.S. Patent Nos. 5,565,332; 4,946,778; 5,648,237; 5,667,988; 4,816,567; Haseman *et al.* (1990) *Proc. Natl. Acad. Sci. USA 87*:3942-3946; Huse *et al.* (1989) *Science 246*:1275-1281; McCafferty *et al.* (1990) *Nature 348*:552-554; Horwitz *et al.* (1988) *Proc. Natl. Acad. Sci. USA 85*:8678-8682; Cabilly *et al.* (1984) *Proc. Natl. Acad. Sci. USA 81*:3273-3277; Horwitz (1989) *Methods in Enzymology 178*:476-496; and Condra *et al.* (1990) *J. Biol. Chem. 265*:2292-2295; the disclosures of which are herein incorporated by reference.

Antibody expression clones express fusion proteins comprising single chain antibodies. Use of such clones for expressing single chain antibodies is taught, for example, in G.P. Smith *et al.* (1985) *Science 228*:1315; S.F. Parmley & G.P. Smith *et al.* (1988) *Gene 73*:305; Soderlind *et al.* (1995) *Gene 160*:269; Calcutt *et al.* (1993) *Gene 137*:77; Pilkington *et al.* (1996) *Mol. Immunol. 33*:4399; Yamanaka *et al.* (1996) *J. Immunol. 157*:1156; and Krebber *et al.* (1997) *J. Immunol. Meth. 201*:35, which are herein incorporated by reference. The clones can be, for example, bacteriophage or phagemids which have been genetically engineered so as

to express the single chain antibodies. Phagemids are plasmids which contain an origin of expression derived from a filamentous bacteriophage (see Sambrook *et al.* (1989) *Molecular Cloning*, pp. 4.17-4.20). Intracellularly expressed antibodies are referred to as "intrabodies." See, for example, Chen *et al.* (1994) *Hum. Gene. Ther. 5*:595-601; Marasco *et al.* (1995) *Immunotechnology 1*:1-19; Rondon *et al.* (1997) *Annu. Rev. Microbiol. 51*:257-283; and von Mehren *et al.* (1996) *Curr. Opin. Oncol. 8*:493-498; herein incorporated by reference. The antibody expression clones can also be bacteria which express single chain antibodies. The clones are constructed using cDNA encoding single chain antibodies which are immunoreactive with proteins of one of the two cells to be compared. The single chain antibodies are encoded by recombinant cDNA molecules which are constructed as follows.

Whole cells or cell lysates of one of the two cell types are used to immunize an animal such as a mouse, rabbit, goat, pig, or other animal, using standard techniques known in the art. Immunization protocols can be used which will enrich the population of B cells for those cells secreting antibodies which are immunoreactive with particular cell types. These protocols enrich the number of antibodies which bind to antigens of interest and also reduces non-specific background binding in the present invention. For example, one can select for B cells which secrete antibodies immunoreactive with cells having a high probability of metastasizing (for example, melanoma cells) but not with cells having a low probability of metastasizing (for example, weakly metastatic cells such as basal cell carcinomas of the skin). In one such protocol an animal, such as a mouse, is injected prenatally with weakly metastatic human cancer cells. Thereafter, the developing mouse recognizes antigens of the weakly metastatic cells as "self." Three to four weeks later, the same mouse is injected with highly metastatic human cancer cells. The mouse will preferentially generate B cells which react with antigens of the highly metastatic cells and which are not found on the weakly metastatic cells.

Alternatively, an adult animal can be injected with weakly metastatic human cancer cells. The animal is then given a drug, for example an alkylating agent such as cyclophosphamide, chlorambucil, busulfan, melphalan, β-propriolactone,

dimethylsulfate, or diepoxybutane, to kill proliferating B cells. This procedure can be repeated two or three times. The animal is then injected with highly metastatic human cancer cells. This process enriches the number of B cells which secrete antibodies immunoreactive with highly metastatic cells, while reducing the number of other B cells present in the animal under normal conditions. Similar procedures can be used to enrich B cell populations for cells which preferentially secrete antibodies against other cell types.

Additionally, the B cells generated using any of these methods can be sorted after isolation from the immunized animal, for example by FACS sorting to separate B cells with surface markers which indicate activated B cells generated by the recent immunization from other B cells.

B cells from the immunized animal can be isolated and used to form hybridomas using techniques known in the art. Messenger RNA is purified using standard techniques from either the B cells or the resulting hybridomas. Reverse transcriptase is then used to generate first strand cDNA from the total isolated mRNA. Those of skill in the art can select suitable primers from sequences of the constant regions flanking the antibody heavy and light chain variable regions and use the primers to amplify these regions from the cDNA using the polymerase chain reaction (PCR). Complementary DNA molecules encoding single chain antibodies are then constructed by ligating a cDNA molecule encoding a variable heavy chain to a cDNA encoding a variable light chain using a linker sequence. PCR amplification is carried out on the cDNA using primers preferably containing restriction sites at their 5′ ends. The final cDNA constructs, which encode single chain antibodies immunoreactive with protein antigens from the immunizing cell type can be used to construct antibody expression clones which will express fusion proteins comprising the single chain antibodies.

The single chain antibodies to be expressed by the antibody expression clones can also be constructed synthetically, for example, by starting with a cDNA molecule encoding a constant framework region of an antibody. Oligonucleotide sequences which encode all possible combinations of peptides 6-15 amino acids in length can be synthesized using standard methods. These oligonucleotides can be combined with the constant framework region to form single chain antibodies

which will recognize epitopes which may not be recognized by the single chain antibodies derived from mRNA of an immunized animal.

In one preferred embodiment, bacteriophage or phagemids are used to produce antibody expression clones. Bacteriophage or phagemids of the antibody expression clones used in the present invention preferably express fusion proteins which comprise single chain antibodies fused to a bacteriophage surface protein. Standard recombinant DNA techniques can be used to construct cDNA molecules which encode this fusion protein, for example by ligating cDNA encoding a single chain antibody with a DNA encoding a phage surface protein such as the gene III product of the bacteriophage M13. Preferably, the cDNA encoding the single chain antibody is less than 335 base pairs in length. The DNA encoding the fusion protein is cloned into the bacteriophage or phagemid using techniques known in the art (see, for example, WO 92/01047, incorporated herein by reference). Infected bacteria are plated on agar containing the appropriate nutrient mixture for the *E. coli* strain and allowed to secrete the bacteriophage or phagemids, forming plaques. Alternatively, bacteria can be transfected with expression constructs encoding single chain antibodies by standard methods and used to form colonies of antibody expression clones.

Antibody expression clones in the plaques can be transferred to a solid support, including, but not limited to, filter membranes (such as nitrocellulose, nylon, or paper), glass or plastic slides or tissue culture plates, beads (such as latex, polyvinylchloride, or polystyrene beads), natural or synthetic fabrics, or silicon-based supports. After transfer, the solid support contains a pattern of antibody expression clones which express single chain antibodies which ar immunoreactive with proteins in one of the cells to be compared.

The proteins to be labeled and compared can be obtained from freshly isolated tissues or from cell lines. Any label known in the art can be used, including but not limited to radioisotopes and biotinylated labels. The proteins can be labeled *in vitro* or, in the case of experimental organisms other than humans, *in vivo*. Proteins are labeled *in vitro*, for example, by exposing cells to labeled amino acids under culture conditions whereby the cells incorporate the labeled amino acids into proteins. Such methods are widely known in the art. Alternatively,

experimental animals can be given food containing labeled amino acids. The labeled amino acids are incorporated into proteins. The cells containing the labeled proteins can be lysed by any means known in the art, such as osmotic shock, sonification, use of a French press or tissue homogenizer, blending with glass beads, or freezing the cells with liquid nitrogen followed by blending. Preparations of labeled proteins from the lysed cells can be obtained using standard biochemical methods.

A labeled protein preparation from a first cell can be contacted with a solid support comprising the pattern of antibody expression clones. The contacting step is carried out under conditions where the single chain antibodies expressed by the antibody expression clones will bind specifically to protein antigens in the protein preparations, for example in the presence of suitable blocking serum. A first pattern of immunoreactivity is determined. The first pattern of immunoreactivity results from the binding of labeled proteins from the first cell with the single chain antibodies of the antibody expression clones. After the contacting step, the solid support can be treated to remove unbound or non-specifically bound proteins. For example, the solid support can be rinsed one or more times in a buffer which does not contain proteins.

Plaques or colonies which contain labeled proteins bound to single chain antibodies can be visualized by methods known in the art suited for detecting the particular protein label being used. For example, radiolabeled proteins bound to the single chain antibodies can be visualized by exposure to X-ray film. Biotinylated proteins can be visualized by incubation with a streptavidin conjugate with subsequent exposure to a detection system. Antibody expression clones comprising single chain antibodies which have bound labeled proteins specifically display a signal at least two-, five-, ten-, twenty-, or fifty-fold higher than those to which labeled proteins have bound non-specifically. After treating to visualize the bound proteins, the solid support displays a pattern of immunoreactivity, indicating the location of antibody expression clones which express single chain antibodies which are bound to the labeled proteins. If desired, the pattern of immunoreactivity can be photographed or scanned into a computer to facilitate

comparison with a pattern of immunoreactivity of labeled proteins of the second cell.

A labeled protein preparation from the second cell, to which proteins in the first cell will be compared, can be contacted with a second solid support which comprises a pattern of antibody expression clones which is identical to the pattern of antibody expression clones on the first solid support. Alternatively, the first solid support can be treated to remove the bound labeled proteins of the first cell, for example by washing with a high salt buffer. Labeled proteins of the second cell can then be contacted with the treated support. A second pattern of immunoreactivity can then be determined and visualized, as described above.

The patterns of immunoreactivity from the first and second cells can be compared, for example, by visual observation or computer-assisted comparison of the solid supports themselves, or photographs or computer-generated images of the supports. A differentially expressed protein will be identified by the presence or absence of a labeled spot in one of the patterns of immunoreactivity. The labeled spot corresponds to an antibody expression clone which expresses a single chain antibody. The single chain antibody is identified as binding to a protein which is differentially expressed between the two cells that were compared.

In one embodiment, the solid support is a filter, such as a nitrocellulose or nylon filter. Colonies or plaques of antibody expression clones can be transferred from plates containing the growing clones to the filters using methods known in the art. Preferably, duplicate filter transfers are performed, and the filters are marked so they can be aligned for comparison after contacting preparations of labeled proteins from the first and second cells. Individual plaques or colonies identified as containing a clone of interest can be selected by reference to their position on the filter and the corresponding position on the plate containing the growing clones. Bacteriophage or bacteria in the plaque or colony can then be isolated and cultured, and the single chain antibody of interest can be retrieved form the culture using standard methods.

In another embodiment, the solid support comprises a pattern of beads. The beads can be any beads to which proteins or nucleic acids can be attached, including, but not limited to, latex, polystyrene, or polyvinylcholoride beads. The

solid support can be, for example, a glass or plastic slide or tissue culture plate, such as a 6-, 12-, 24-, 48-, or 96-well plate. The beads can be attached to the solid support by using an adhesive, as is known in the art. Alternatively, the beads can be placed without attachment in compartments on the solid support.

Antibody expression clones which express single chain antibodies, such as bacteriophage, can be bound to the beads using a stable but reversible chemical linkage, *e.g.*, a disulfide bond, between a protein on the surface of the antibody expression clone and the surface of the bead. Cross-linkers with spacer arms of varying lengths, such as 1-(*p*-azidosalicylamido)-4-(iodoacetamido)butane. 1-ethyl-3-(3-dimethylaminopropyl)carbodimide hydrochloride, *N*-hydroxylsulfosuccinimidyl 4-azidobenzoate sulfosuccinimidyl 4-(*p*-azidophenyl)butyrate, sulfosuccinimidyl β-[alpha-methyl-alpha-(2-pyridyldithio)toluamido]hexanoate, 1,4-di-[3'-(d'-pyridyldithio)propionamido]butane, sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide) ethyl-1,3'-dithiopriopionate, or sulfo-succinimidyl 7-azido-4-methylcoumarin-3-acetate, can also be used to attach single chain antibodies to a bead. The bead can optionally be coated with a substance such as laminin, polylysine or polyornithine, to facilitate such linkage. The beads can be arrayed in an identifiable pattern on the solid support. Identifying labels, such as unique oligonucleotide sequences, can be attached to the beads by a chemical linkage. Each oligonucleotide label can contain a unique primer site for sequencing, so that such labels can be sequenced and identified.

After identifying a bead or beads bound to single chain antibodies which bind proteins which are differentially expressed between the two compared cells, the single chain antibodies can be removed from the beads by breaking the reversible chemical linkages by which they were attached. Alternatively, they can be retrieved from a separately studied sample.

The single chain antibody corresponding, for example, to a labeled plaque, colony or identified bead, can be isolated and used to screen a population of protein expression clones which express cDNA of the first cell. To construct the protein expression clones, total mRNA is isolated from the first cell using methods known in the art. Reverse transcriptase can be used to transcribe the isolated mRNA into cDNA. The cDNA thus obtained by reverse transcription of the

isolated mRNA encodes proteins which are expressed by the first cell. These proteins include those which are differentially expressed between the first cell and the second cell.

Using standard recombinant DNA techniques, the cDNA from the first cell can be introduced into a cloning vector to form protein expression clones. A variety of cloning vectors for use with protein expression systems are available in the art, including but not limited to bacteriophage for use in bacterial expression systems, retroviral vectors for use in mammalian expression systems, or baculoviral vectors for use in insect expression systems. In a preferred embodiment, the cloning vector is a λgt11 bacteriophage and the host cell is an *E. coli* Y1090 or BNN97 cell.

Host cells comprising the cDNA-containing cloning vectors can be plated onto a suitable medium, forming protein expression clones. The protein expression clones can be allowed to form plaques or colonies, which can be transferred to a solid support. In a preferred embodiment, the solid support is a nylon or nitrocellulose filter. After the transfer, the solid support contains a pattern of protein expression clones which contain cDNA molecules which encode proteins of the first cell.

The identified single chain antibody can be labeled, for example with a radioisotopic, fluorescent, or enzymatic label, and contacted with the solid support comprising the pattern of protein expression clones. The step of contacting is carried out under conditions where the single chain antibody will bind specifically to a protein expressed from a cDNA molecule which is localized in a particular plaque on the solid support. Single chain antibodies which bind proteins specifically display a signal at least two-, five-, ten-, twenty-, or fifty-fold higher than those to which labeled proteins have bound non-specifically. Plaques or colonies containing bound labeled proteins are detected as described above. Labeled plaques or colonies are identified as containing a protein expression clone which contains DNA from a gene which is differentially expressed between the first and the second cell. That DNA can be sequenced and, if desired, used to express the protein which is differentially expressed. Genomic DNA encoding the protein can also be isolated using the DNA of the protein expression clone.

Solid supports comprising a pattern of antibody expression clones according to the present invention are also provided in a kit. As described above, the solid supports may be, for example, filter membranes (such as nitrocellulose, nylon, or paper), glass or plastic slides or tissue culture plates, beads (such as glass, sepharose, cellulose, latex, polyvinylchloride, or polystyrene beads), natural or synthetic fabrics, or silicon-based supports. The antibody expression clones express fusion proteins comprising single chain antibodies against proteins of a cell population. Preferably, the single chain antibodies are immunoreactive with proteins in a non-diseased cell. The proteins can be obtained from any mammalian cell or tissue, such as mammary gland, colon, stomach, brain, liver, lung, pancreas, reproductive system, skin, thymus, skeletal muscle, peripheral blood lymphocytes, placenta, kidney, lymph node, or prostate. The cell can be at any developmental stage, including embryonic, fetal, juvenile, or adult stages. Preferably, the kit also contains a viable sample of the cell population which was used to obtain the antibodies from which the singe chain antibodies were derived. This sample can be used for isolating proteins which are expressed in that cell population but not in the population of cells which were tested using the kit.

A kit is also provided which contains a first solid support which can be used to identify a single chain antibody which binds to a protein which is differentially expressed between a first cell and a second cell, as described above, and a second solid support comprising a pattern of protein expression clones comprising cDNA obtained by reverse transcription of mRNA isolated from the first cell. The first solid support can be used to identify a single chain antibody which binds to a protein which is differentially expressed between the first cell and a second cell. The second solid support can be screened with the identified single chain antibody to identify a protein expression clone which expresses a cDNA encoding a protein which is differentially expressed between the first cell and the second cell.

If single chain antibodies are expressed intracellularly in a population of cells to be studied, rather than on the surface of an antibody expression clone, a gene which encodes an intracellular protein which participates in a cellular response can be identified. The cellular response can be any response in which there is a

demonstrated interest. These responses include but are not limited to apoptosis, resistance to a pathogen or toxin, alterations in cellular growth, division, or differentiation, motility, chemotaxis, immune response, cell-cell adhesion, or cellular migration.

Animals can be immunized against a lysate of the cell population to be tested, as described above, and B cells expressing antibodies against intracellular proteins in the lysed cells can be obtained. Messenger RNA can be isolated from the B cells and used to construct cDNA molecules which encode single chain antibodies which are immunoreactive with protein antigens of the cell population to be tested. Nucleic acid vectors comprising the cDNA encoding the single chain antibodies can be made using standard recombinant DNA techniques. A variety of nucleic acid vectors are available for this purpose, including but not limited to retroviral and adenoviral vectors for use in mammalian cells, baculoviral vectors for use in insect cells, and bacteriophage vectors for use in bacteria. The vectors can be introduced into the population of cells to be tested using methods known in the art.

The cells to be tested can be any cells capable of being maintained *in vitro*, such as bacteria or cell lines or explant cultures of animal or plant tissues. After their introduction into the cells, the nucleic acid vectors express the single chain antibodies intracellularly, where the single chain antibodies can bind to intracellular proteins of the cells.

The population of cells containing the single chain antibody expression vectors can be contacted with an inducer of a cellular response. For example, the inducer can be Fas ligand and the cellular response can be apoptosis. The inducer can be a pathogen, such as a bacteria, virus, fungus, mycoplasma, protozoan, or prion, and the response can be resistance to the pathogen. The inducer can be a naturally occurring or a synthetic chemical, and the cellular response can be, for example, an alteration in the growth, differentiation, or immune response of the cell.

Intracellular proteins can be identified which participate in a cellular response of a diseased cell to a therapeutic chemical, for example a reduction in growth rate of a neoplastic cell. The inducer induces the cellular response in a first

subset of the population of cells. However, in a second subset of the population of cells, the cellular response is not induced because one of the single chain antibodies has bound to an intracellular protein which participates in the cellular response.

The first and the second subset of cells can be separated on the basis of an appropriate characteristic for the cellular response being examined. Separation can be based on morphology, differential adherence to the culture surface, lytic response to a pathogen, or a variety of other characteristics which will be clear to those skilled in the art based on the desired cellular response. The second subset of the population of cells can be lysed by any means known in the art, as described above. At least one nucleic acid vector encoding a single chain antibody can then be isolated from the second subset of the population of cells and used to express the single chain antibody it encodes. Such isolation and expression can be accomplished using standard techniques.

The single chain antibody can be labeled and used to screen a population of protein expression clones. Preferably the antibody is labeled, although it can be detected otherwise, *e.g.*, using a second antibody. The population of expression clones contain cDNA obtained by reverse transcription of mRNA isolated from the same type of cells. A protein expression clone which directs expression of an intracellular protein which binds to a single chain antibody is identified as encoding an intracellular protein which is involved in the cellular response in the population of cells.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Preparation of Antibody Libraries

To prepare a library of human-derived antibody genes, synthetic consensus sequences that cover the structural repertoire of antibodies encoded in the human genome can be utilized. See, for example, PCT/EP 96/03647, herein incorporated by reference. The method utilizes a single consensus antibody gene as a universal framework for highly diverse antibody libraries. The method provides for the

creation of libraries of human antibodies, wherein said polypeptides are derived from heavy or light chain variable regions wherein said structural sub-elements are framework regions, 1, 2, 3, or 4 are complementary determining regions 1, 2, or 3.

Alternatively, diverse libraries of immunoglobulin heavy (V_H) and light (V_K and V_λ) chain variable (V) genes are prepared from peripheral blood, lymphocytes of unimmunized donors by polymerase chain reaction amplification. Genes encoding single chain Sv fragments are made by randomly combining heavy and light chain V-genes using PCR, and the combinatorial library is cloned for display on the surface of a phage. See, for example, U.S. Patent No. 5,565,332, and PCT Application WO 92/01047, herein incorporated by reference.

The use of standard phage display systems for optimizing antibodies for a given target protein are known in the art. See, for example, Krabber 91997) *J. Immunol. Meth.* 201:35-55; Wamanaka, *et al.* (1996) *J. Immunol.* 157:1156-1162; Tsurushita, *et al.* (1996) *Gene* 172:59-63; Pilkington, *et al.* (1996) *Mol. Immunol.* 33:439-450; Merz, *et al.*, (1995) *J. NeuroScience Meth.* 62:213-219; the disclosures of which are herein incorporated by reference.

Plating and Transferring BacterioPhage Libraries

Bacteriophage are plated onto agar plates at high density. The recombinant phage is mixed with plating bacteria in a culture tube and incubated 20 minutes at 37°C. (In Quest and Sternberg (1979) *Meth. Enzymol.* 68:281-298; and Stent, G. S. (1971) *Molecular Genetics: An Introductory Narrative*, W. H. Freeman, NY). 0.7% top agarose is added to the culture tube and the mixture transferred to LB plates. The bacteria and agarose is dispersed onto plates (the top agarose is melted and cooled to 45° to 50°C) before use. Plates are incubated at 37°C until plaques cover the plate but are not confluent. Incubation time may vary between 6 and 12 hours depending on the type of phage and bacteria used. Plates can then be stored at 4°C.

Nitrocellulose filters are labeled with a ballpoint pen and applied face down on cold LB plates bearing bacteriophage plaques as described above. The filters are left on the plates for about five minutes to allow transfer of phage particles to

the filter. During the transfer period, the orientation of the filter to the plate is recorded by, for example, stabbing a needle through the filter into the agar at several asymmetric points around the edge of the plate. At least two replicas are made from each plate. The filters are slowly removed from the plates with blunt, flat forceps and placed face up on paper towels or filter paper. The filters are allowed to dry for at least 10 minutes. The drying process binds the plaques to the filter.

When nitrocellulose is applied to the agarose, phage particles and unpackaged DNA absorbed to the filter to produce a replica of the plate surface. If the agarose surface is not accessibly wet, there will be little spreading of the phage on the filter. After binding to the filter, the filter is treated with sodium hydroxide which destroys the phage particles and denatures the phage DNA which then binds to the nitrocellulose. The filters are placed on three MM paper and saturated with 0.2M Na OH/1.5M NaCl. Place filters on the paper face up for 1 to 2 minutes. Filters are transferred to 3 MM paper saturated with 0.4M Tris-Cl pH 7.6/2x SSC for 1 to 2 minutes and then to 3 MM paper saturated with 2x SSC for 1 to 2 minutes. Filters are dried in a vacuum oven for two hours at 80°C.

Screening of Expression Libraries

A single colony of *E. coli* strain Y1090 *hsdR*, is used as an inoculum to prepare a plating culture. The number of plates that will need to be screened can be calculated by $2x10^4$ plaques per 90-MM plate or $5x10^4$ plaques per 150-MM plate. A set of sterile tubes is arranged in a rack. In each tube, 0.1ml of the plating bacteria is mixed with 0.1ml of SM containing $3x10^4$ pfu (90-MM plates) of the bacteriophage λexpression library. The infected bacteria is incubated for 20 minutes at 37°C. 2.5ml of molten top agarose is added to each tube and the mixture poured onto LB agar plates. The infected plates are incubated for 3.5 hours at 42°C. Nitrocellulose filters are numbered with a soft-lead pencil. The filters are handled with gloved hands. The filters are soaked in a solution of isopropylthio-ß-*D*-glacatoside (IPTG) (10mM in distilled water) for 10 minutes. Using blunt-ended forceps, the filters are removed from the solution and allowed to dry at room temperature. The plates which have been allowed to incubat_ for 3.5

hours are overlaid with the nitrocellulose filters. The plate is then incubated for at least 4 hours at 37°C. Duplicate filters are prepared by removing the first filter after 4 hours and replacing it with a second numbered filter that has been impregnated with IPTG. The plate is then incubated for a further 4-6 hours at 37°C after which the second filter is removed.

Filters are peeled off the plates and immediately immersed in a large volume of TNT (10mM Tris-Cl (pH 8.0); 150mM NaCl; and 0.05% Tween 20). The filters are transferred individually to glass trays containing blocking buffer (20% fetal bovine serum in TNT). The filters are incubated for 30 minutes at room temperature.

The filters are transferred to fresh glass trays containing labeled protein diluted in blocking buffer.

Labeled protein is prepared from two sources, normal cells and tumor cells. Differentially expressed proteins can be detected by a comparison of binding of the labeled protein to immobilize antibody.

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Using the methods described above, specific peptide-phages are panned against purified recombinant target proteins such as, for example, epithelial growth factor receptor (EGF-R) and platelet-derived growth factor receptor (PDGF-R), by conventional methods. Panning can be conducted, for example, as described in the following, including the references cited in these references: Wrighton *et al.* (1996) *Science* 273:458-463; Cwirla *et al.* (1997) *Science* 276:1696-1699; Cesareni (1992) *FEBS Lett.* July 27; 307(1):66-70; Desai *et al.* (1998) *Cancer Res.* 58(11):2417-2425; Carcamo *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95(19):11146-11151; Gardsvoll *et al.* (1998) *FEBS Lett.* 431(2):170-174; Williams *et al.* (1998) *J. Immunol. Methods* 213(1):1-17; and Giersch *et al.* (1994) *Curr. Biol.* 4(2):173-174.

The first set of experiments is illustrated in Table 1. Increasing amounts of recombinant target proteins will be separated by SDS-PAGE and Western-blotted onto nitrocellulose membranes. Individual membranes are probed with peptide-phages against, for example, either EGF-R(B) or PDGF-R(C). Binding affinity and

specificity of the peptide phages to the individual target proteins is visualized by a secondary antibody against M13 phage particle linked to alkaline phosphatase. Table 1 shows that peptide-phages bind with high specificity. Affinities of specific peptide-phages to target protein receptors are expected to differ.

Table 1. Immuno blot-type experiment using specific peptide-phages against specific target protein receptors.

A. Peptide-Phage against EGF-R			
Target Protein	Recombinant		Protein
	1	10	100 ng
EGF-R	----	+	++
PDGF-R	----	----	----

B. Peptide-Phage against PDGF-R			
Target Protein	Recombinant		Protein
	1	10	100 ng
EGF-R	----	----	----
PDGF-R	+++	+++	+++

The second set of experiments is illustrated in Figure 1. Peptide-phages are immobilized using a monoclonal mouse-anti M13 antibody and incubated with a cell lysate or a mixture of different recombinant proteins including the target protein receptor. After extensive washing using standard techniques, the bound target protein receptor will be identified using a specific antibody against the target protein receptor linked to a detection molecule, for example alkaline phosphatase (goat antibody to target protein receptor) for detection.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED:

1. A method for identifying an antibody which binds to a differentially expressed protein, comprising the steps of:

(a) contacting labeled proteins from a first cell with a first solid support under conditions where antibodies specifically bind to proteins, wherein the solid support comprises a pattern of antibody expression clones, wherein the antibody expression clones express fusion proteins comprising single chain antibodies, wherein the single chain antibodies are immunoreactive with proteins of the first cell;

(b) determining a first pattern of immunoreactivity on the first solid support formed by the binding of labeled proteins from the first cell with the single chain antibodies expressed by the antibody expression clones;

(c) contacting labeled proteins from a second cell with a second solid support comprising the pattern of antibody expression clones;

(d) determining a second pattern of immunoreactivity on the second solid support formed by the binding of labeled proteins from the second cell with the single chain antibodies expressed by the antibody expression clones; and

(e) comparing the first pattern of immunoreactivity with the second pattern of immunoreactivity, wherein a difference between the first and second patterns of immunoreactivity identifies an antibody which binds to a protein which is differentially expressed between the first cell and the second cell.

2. The method of claim 1, wherein the first and second solid supports comprise identical patterns of antibody expression clones.

3. The method of claim 1, wherein the second solid support is the first solid support which has been treated to remove bound labeled proteins from the first cell.

4. The method of claim 1, wherein the antibody expression clones are bacteriophage.

5. The method of claim 1, wherein the antibody expression clones are bacteria.

6. The method of claim 1, wherein the solid supports comprise a pattern of beads, wherein the antibody expression clones are bound to the beads.

7. The method of claim 6, wherein the beads are reversibly attached to the solid support.

8. The method of claim 6, wherein identifying labels are attached to the beads.

9. The method of claim 1, wherein the single chain antibodies are expressed on the surface of the antibody expression clones.

10. The method of claim 1, wherein either the first or the second cell is a neoplastic cell, wherein if the first cell is a neoplastic cell then the second cell is a non-neoplastic cell, and if the second cell is a neoplastic cell then the first cell is a non-neoplastic cell.

11. The method of claim 1, wherein either the first or the second cell is a highly metastatic cell, wherein if the first cell is a highly metastatic cell then the second cell is a weakly metastatic cell, and if the second cell is a highly metastatic cell then the first cell is a weakly metastatic cell.

12. The method of claim 1, wherein either the first or the second cell is infected with a pathogen, wherein if the first cell is infected with the pathogen then the second cell is not infected with the pathogen, and if the second cell is infected with the pathogen then the first cell is not infected with the pathogen.

13. The method of claim 1, wherein either the first or the second cell is a diseased cell, wherein if the first cell is a diseased cell then the second cell is a

non-diseased cell, and if the second cell is a diseased cell then the first cell is a non-diseased cell.

14. The method of claim 1, wherein the first cell is at a first developmental stage and the second cell is at a second developmental stage.

15. The method of claim 1, wherein either the first or the second cell is exposed to an environmental factor, wherein if the first cell is exposed to the environmental factor then the second cell is not exposed to the environmental factor, and if the second cell is exposed to the environmental factor then the first cell is not exposed to the environmental factor.

16. The method of claim 1, wherein either the first or the second cell is contacted with an exogenous substance, wherein if the first cell is contacted with the exogenous substance then the second cell is not contacted with the exogenous substance, and if the second cell is contacted with the exogenous substance then the first cell is not contacted with the exogenous substance.

17. The method of claim 1, wherein the first cell originates from a first tissue type and the second cell originates from a second tissue type.

18. The method of claim 1, wherein the first cell originates from a first species and the second cell originates from a second species.

19. The method of claim 1, wherein the labeled proteins are radioactively labeled.

20. The method of claim 1, wherein the labeled proteins are biotinylated.

21. A method for identifying a gene which is differentially expressed between two cells comprising the steps of:

(a) identifying a single chain antibody which binds to a protein which is differentially expressed between two cells according to the method of claim 1;

(b) isolating the identified single chain antibody; and

(c) using the single chain antibody to screen a population of protein expression clones comprising cDNA obtained by reverse transcription of mRNA isolated from the first cell, wherein a protein expression clone which binds to the single chain antibody is identified as expressing a cDNA encoding a protein which is differentially expressed between the two cells.

22. A solid support comprising a pattern of antibody expression clones, wherein the antibody expression clones express fusion proteins comprising single chain antibodies.

23. The solid support of claim 22, wherein the single chain antibodies are immunoreactive with proteins in a non-diseased cell.

24. A kit comprising at least two solid supports according to claim 22.

25. The kit of claim 24 further comprising a viable sample of the cell population.

26. A kit for identifying a gene which is differentially expressed between a first cell and a second cell, comprising:

(a) a first solid support comprising a pattern of antibody expression clones, wherein the antibody expression clones express fusion proteins comprising single chain antibodies, wherein the single chain antibodies are immunoreactive with proteins of the first cell, wherein the first solid support can be used to identify a single chain antibody which binds to a protein which is differentially expressed between a first cell and a second cell; and

(b) a second solid support comprising a pattern of protein expression clones comprising CDNA obtained by reverse transcription of mRNA

isolated from the first cell, wherein the protein expression clones express cDNA encoding proteins of the first cell, wherein the second solid support can be screened with the identified single chain antibody to identify a protein expression clone which expresses a cDNA encoding a protein which is differentially expressed between the first cell and the second cell.

27. The kit of claim 26 further comprising a viable sample of the first cell for preparing proteins to screen the single chain antibodies.

28. A method of identifying a gene which encodes an intracellular protein which participates in a cellular response, comprising the steps of:

(a) introducing a library of nucleic acid vectors encoding single chain antibodies into a population of cells, wherein the vectors express single chain antibodies intracellularly, wherein the single chain antibodies bind to intracellular proteins of the population of cells;

(b) contacting the population of cells with an inducer of a cellular response, wherein the inducer induces the cellular response in a first subset of the population of cells but does not induce the cellular response in a second subset of the population of cells;

(c) isolating at least one nucleic acid vector encoding a single chain antibody from the second subset of the population of cells and expressing the single chain antibody; and

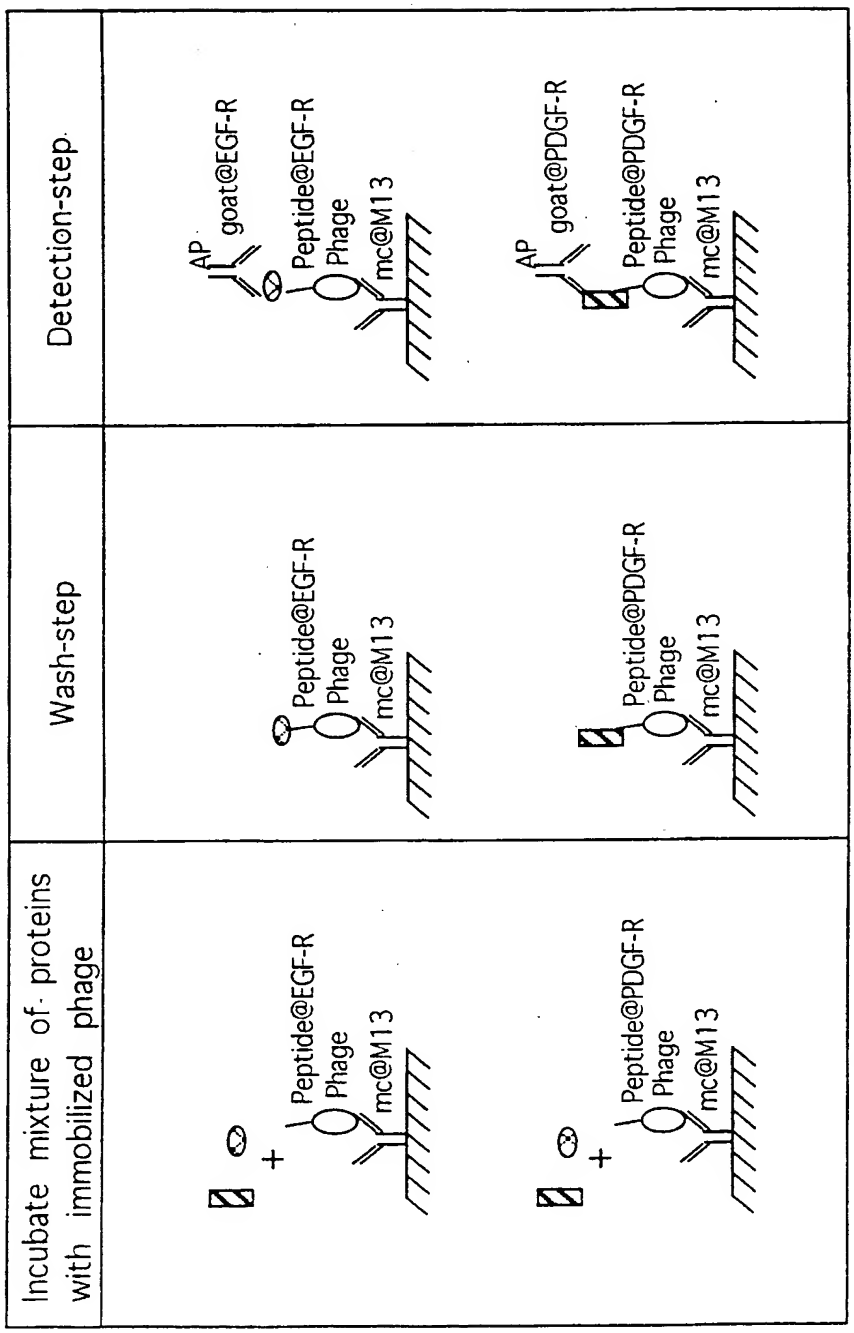
(d) using the expressed single chain antibody encoded by the isolated vector to screen a population of protein expression clones comprising cDNA obtained by reverse transcription of mRNA isolated from the population of cells, wherein a protein expression clone which binds to the expressed single chain antibody is identified as expressing a cDNA encoding an intracellular protein which is involved in the cellular response in the population of cells.

29. The method of claim 28, wherein the inducer is Fas ligand and the cellular response is apoptosis.

30. The method of claim 28, wherein the inducer is a pathogen and the cellular response is resistance to the pathogen.

31. The method of claim 28, wherein the inducer is a toxin and the cellular response is apoptosis.

Figure 1. ELISA-type experiment using specific immobilized peptide-phages to capture specific target protein receptors.



INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/US 98/19425

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/543 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 1999	24/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Routledge, B

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(43) Date of A Publication 21.06.2000

(21) Application No 9905510.5

(22) Date of Filing 10.03.1999

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(51) INT CL[7]
G01N 33/53 , C07K 19/00 , C12N 15/13 15/62 , G01N 33/542 33/563

(52) UK CL (Edition R)
G1B BAD B103 B108 B121 B122
C3H HB7M H675 H690

(56) Documents Cited
WO 96/40248 A1 **US 4499183 A**
J. Clin. Lab. Anal. 1987 1(1) pp.56-61 - Ahearn et al.

(58) Field of Search
Online: WPI, EPODOC, BIOSIS, SCISEARCH

(54) Abstract Title
Selection of intracellular immunoglobulins

(57) A method for determining the ability of antibody to bind to antigen in an intracellular environment comprises (a) providing first and second molecules which interact to generate a signal; (b) providing an intracellular antibody associated with the first molecule and an intracellular antigen associated with the second molecule; (c) assessing the intracellular Ab/Ag interaction by monitoring the signal generated; and, optionally, (d) isolating those antibodies which give rise to a signal. The antibody may be an intact immunoglobulin or a Fv, scFv, Fab or $F(ab')_2$ fragment. The first molecule may particularly be the activation domain of VP16 and the second molecule may be the binding domain of LexA.

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At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

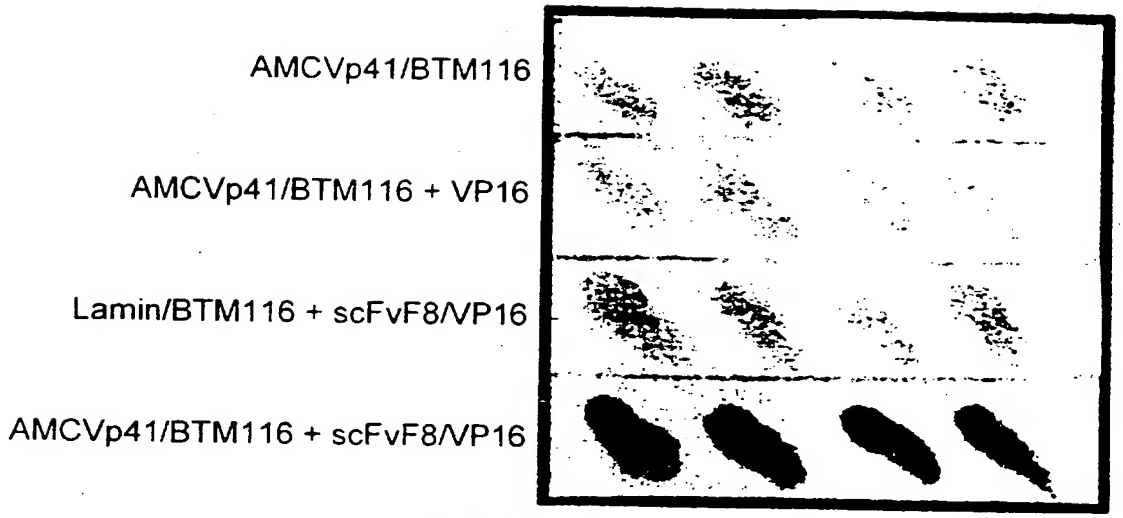


FIG. 1

FIG. 2a

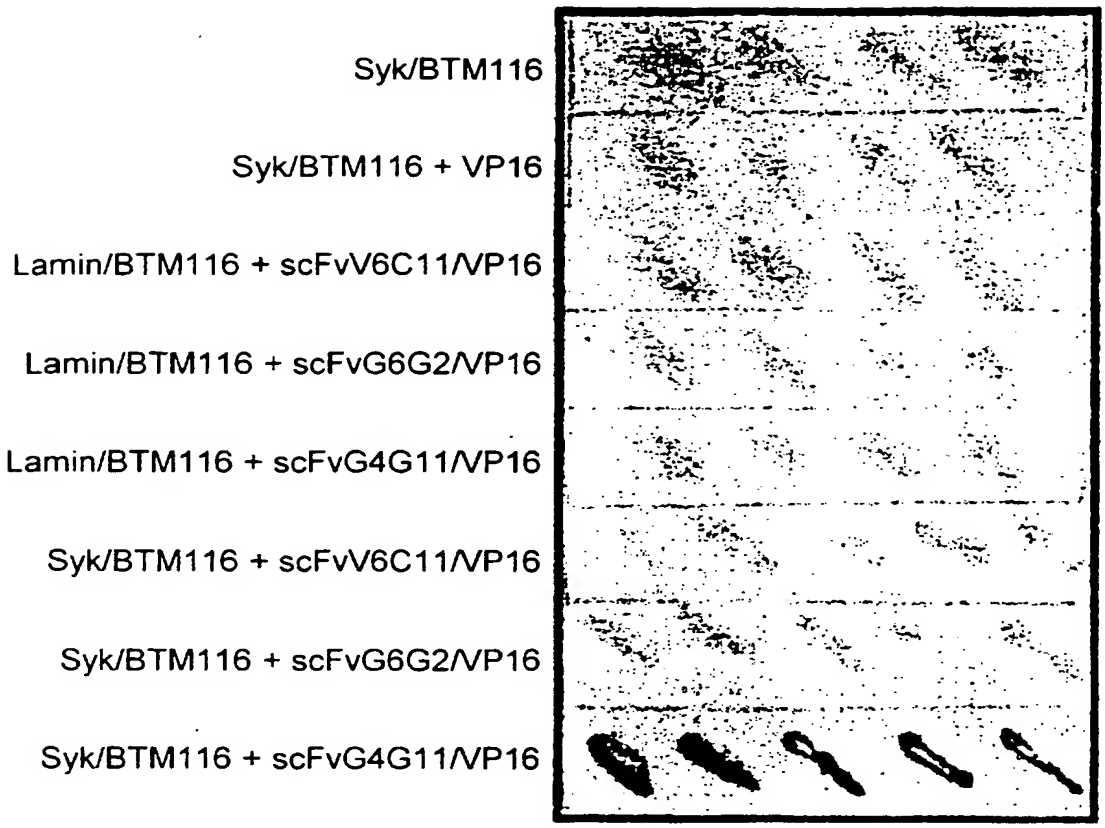


FIG. 2b

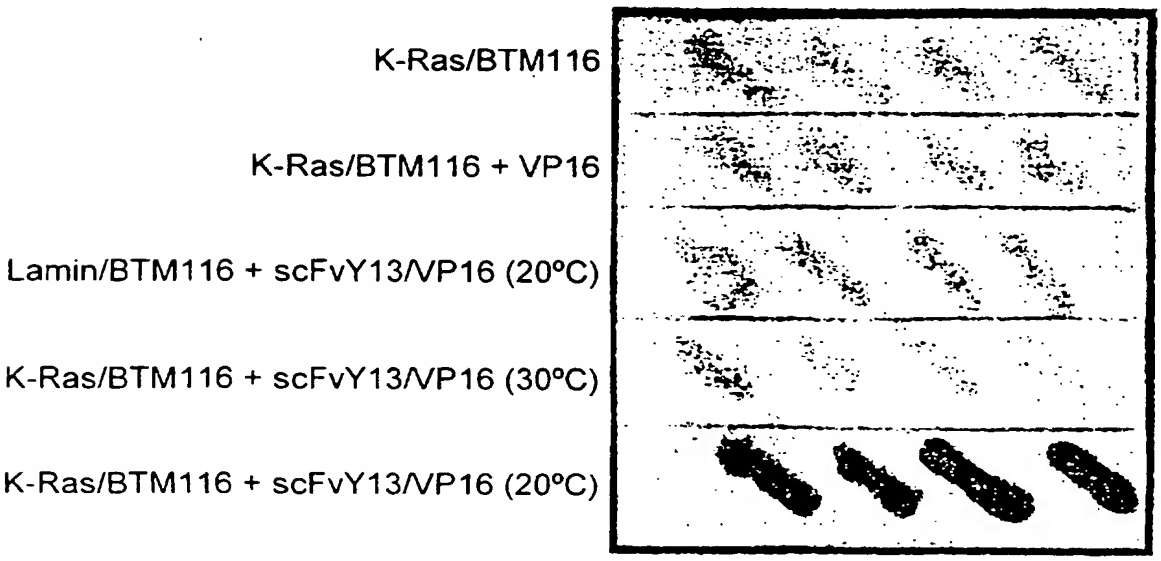


FIG. 2c

A)

	YC-W	YC-WHUK
AMCVp41/BTM116	+	-
K-Ras/BTM116	+	-
Syk/BTM116	+	-
HIV-1 IN/BTM116	+	-
β-gal/BTM116	+	+

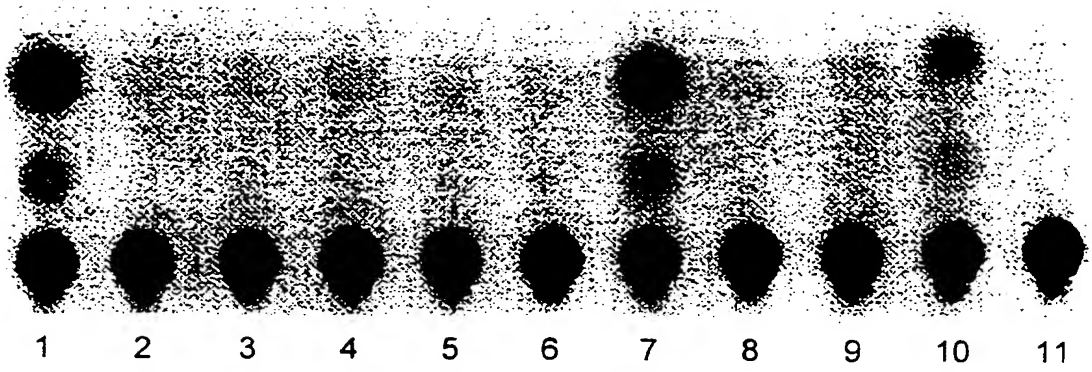
B)

	YC-WL	YC-WHULK
AMCVp41/BTM116 + VP16	+	-
K-Ras/BTM116 + VP16	+	-
Syk/BTM116 + VP16	+	-
HIV-1 IN/BTM116 + VP16	+	-
β-gal/BTM116 +VP16	+	+

C)

"bait"	"prey" scFv/VP16	YC-WL	YC-WHULK
Lamin	F8	+	-
Lamin	Y13	+	-
Lamin	V6C11	+	-
Lamin	G6G2	+	-
Lamin	G4G11	+	-
Lamin	IN33	+	-
Lamin	PM163	+	-
Lamin	PM163R4	+	-

FIG. 3



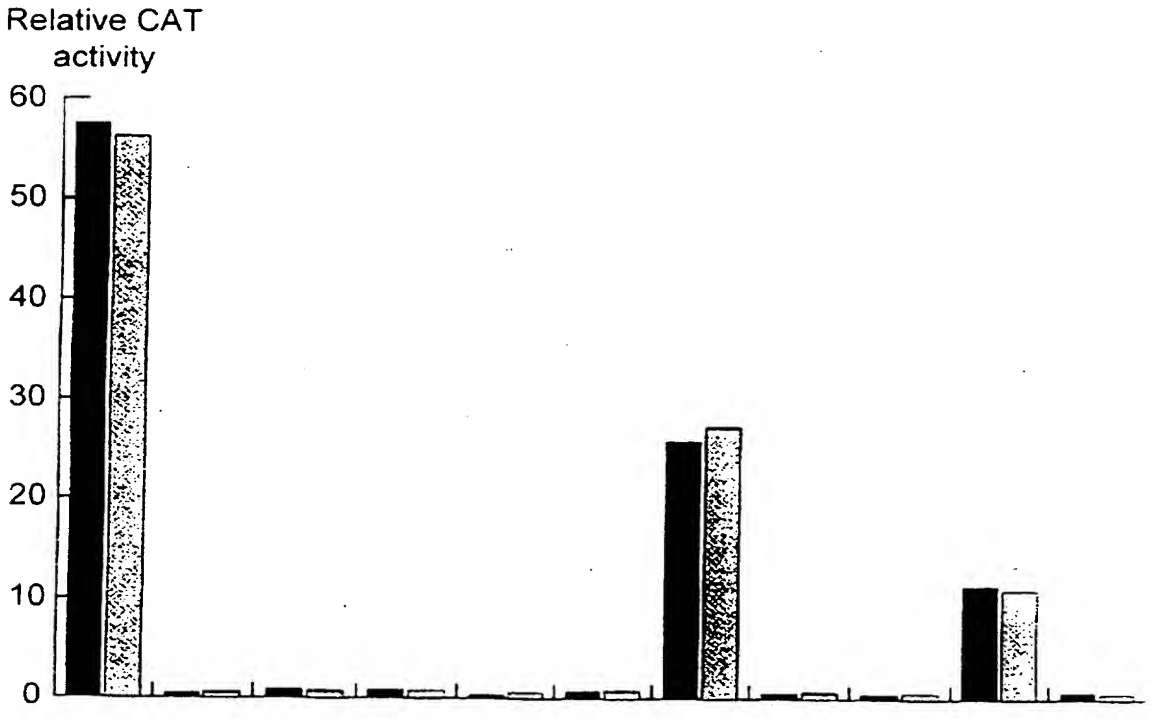


Fig. CAT assay of protein extracts from transiently transfected CHO cells

Lane 1 pM-Bgal + pNLscFvR-VP16
Lane 2 Reporter
Lane 3 pM-Bgal
Lane 4 pNLscFvR4-VP16
Lane 5 pM-Bgal + pNLscFvF8-VP16
Lane 6 pNLscFvR4-VP16 + pM1-AMCV
Lane 7 pM-scFvR4 + pNLVP16-Bgal
Lane 8 pM-scFvR4
Lane 9 pNLVP16-Bgal
Lane 10 pM-Bgal + pNLVP16-scFvR4
Lane 11 pNLVP16-scFvR4

FIG. 4

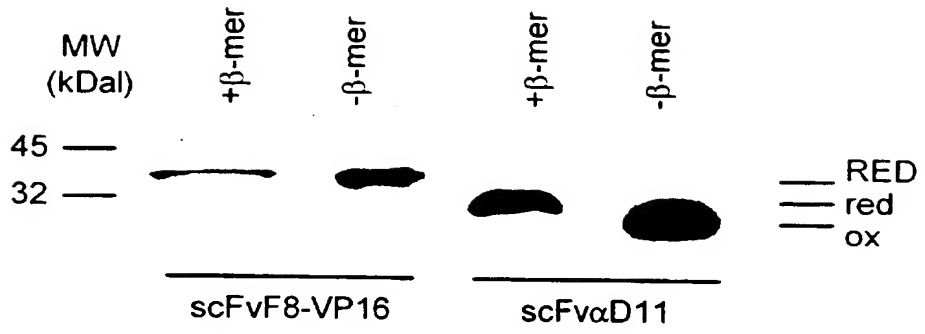


FIG. 5

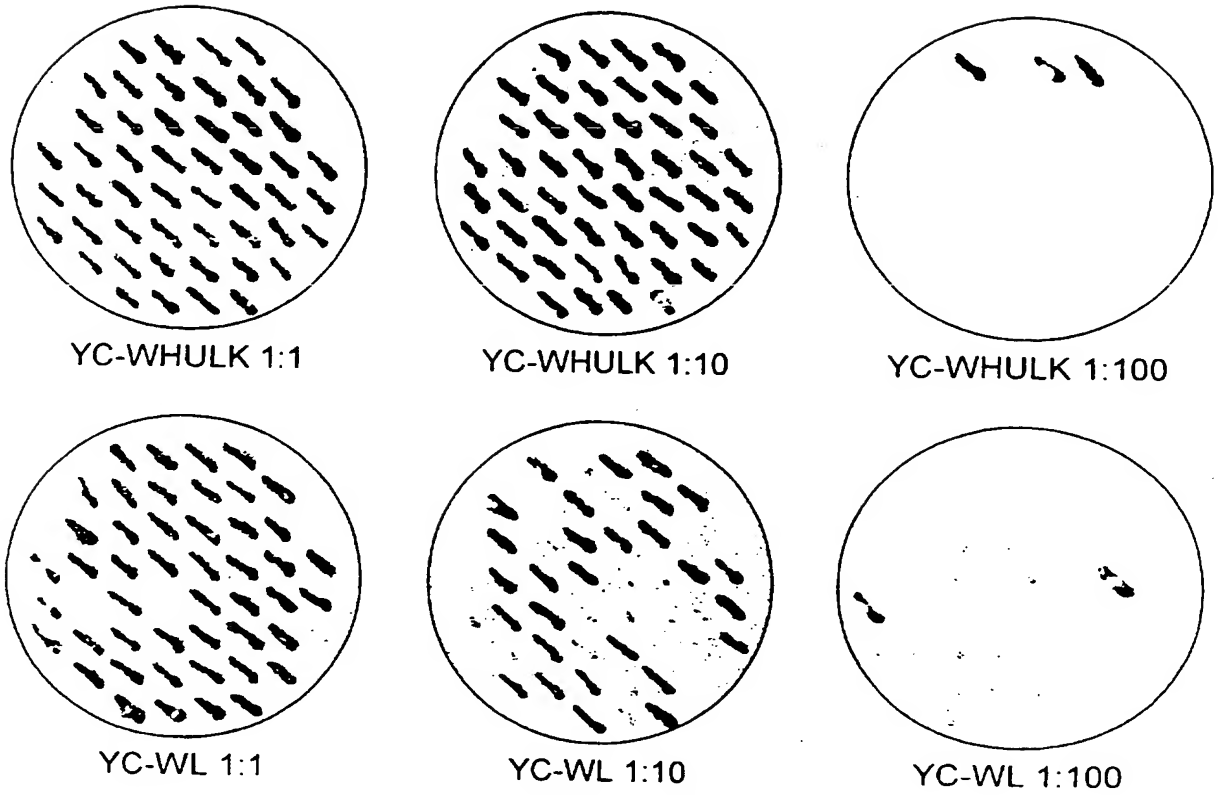


FIG. 6

Intracellular Selection

Field of the Invention

The present invention relates to a method for selecting polypeptides according to their intracellular activity. In particular, the invention relates to a method for selecting immunoglobulin molecules according to their intracellular binding activity, and to the provision of immunoglobulin molecules which are active in an intracellular environment.

Background to the Invention

Intracellular antibodies or intrabodies have been demonstrated to function in antigen recognition in the cells of higher organisms (reviewed in Cattaneo, A. & Biocca, S. (1997) *Intracellular Antibodies: Development and Applications.* Landes and Springer-Verlag). This interaction can influence the function of cellular proteins which have been successfully inhibited in the cytoplasm, the nucleus or in the secretory pathway. This efficacy has been demonstrated for viral resistance in plant biotechnology (Tavladoraki, P., *et al.* (1993) *Nature* **366**: 469-472) and several applications have been reported of intracellular antibodies binding to HIV viral proteins (Mhashilkar, A.M., *et al.* (1995) *EMBO J* **14**: 1542-51; Duan, L. & Pomerantz, R.J. (1994) *Nucleic Acids Res* **22**: 5433-8; Maciejewski, J.P., *et al.* (1995) *Nat Med* **1**: 667-73; Levy-Mintz, P., *et al.* (1996) *J. Virol.* **70**: 8821-8832) and to oncogene products (Biocca, S., Pierandrei-Amaldi, P. & Cattaneo, A. (1993) *Biochem Biophys Res Commun* **197**: 422-7; Biocca, S., Pierandrei-Amaldi, P., Campioni, N. & Cattaneo, A. (1994) *Biotechnology (N Y)* **12**: 396-9; Cochet, O., *et al.* (1998) *Cancer Res* **58**: 1170-6). The latter is an important area because enforced expression of oncogenes often occurs in tumour cells after chromosomal translocations (Rabbitts, T.H. (1994) *Nature* **372**: 143-149). These proteins are therefore important intracellular therapeutic targets (Rabbitts, T.H. (1998) *New Eng. J. Med* **338**: 192-194) which could be inactivated by binding with intracellular antibodies. Finally, the international efforts at whole genome sequencing will produce massive numbers of potential gene sequences which encode proteins about which nothing is known. Functional genomics is an approach to ascertain the function of this plethora of proteins

and the use of intracellular antibodies promises to be an important tool in this endeavour as a conceptually simple approach to knocking-out protein function directly by binding an antibody inside the cell.

Simple approaches to derivation of antibodies which function in cells are therefore necessary if their use is to have any impact on the large number of protein targets. In normal circumstances, the biosynthesis of immunoglobulin occurs into the endoplasmic reticulum for secretion as antibody. However, when antibodies are expressed in the cell cytoplasm (where the redox conditions are unlike those found in the ER) folding and stability problems occur resulting in low expression levels and the limited half-life of antibody domains. These problems are most likely due to the reducing environment of the cell cytoplasm (Hwang, C., Sinskey, A.J. & Lodish, H.F. (1992) *Science* **257**: 1496-502), which hinders the formation of the intrachain disulphide bond of the VH and VL domains (Biocca, S., Ruberti, F., Tafani, M., Pierandrei-Amaldi, P. & Cattaneo, A. (1995) *Biotechnology (N Y)* **13**: 1110-5; Martineau, P., Jones, P. & Winter, G. (1998) *J Mol Biol* **280**: 117-127) important for the stability of the folded protein. However, some scFv have been shown to tolerate the absence of this bond (Proba, K., Honegger, A. & Pluckthun, A. (1997) *J Mol Biol* **265**: 161-72; Proba, K., Worn, A., Honegger, A. & Pluckthun, A. (1998) *J Mol Biol* **275**: 245-53) which presumably depends on the particular primary sequence of the antibody variable regions. No rules or consistent predictions however can yet be made about those antibodies which will tolerate the cell cytoplasm conditions. A further problem is the design of expression formats for intracellular antibodies and much effort has be expended on using scFv in which the VH and VL segments (i.e. the antibody combining site) are linked by a polypeptide linker at the C-terminus of VH and the N-terminus of V_L (Bird, R.E., *et al.* (1988) *Science* **242**: 423-6). While this is the most successful form for intracellular expression, it has a drawback in the lowering of affinity when converting from complete antibody (e.g. from a monoclonal antibody) to a scFv. Thus not all monoclonal antibodies can be made as scFv and maintain function in cells. Finally, different scFv fragments have distinct properties of solubility or propensity to aggregate when expressed in this cellular environment.

There is a need, therefore, to obtain antibody fragments that will fold, are stable and soluble under conditions of intracellular expression. At present, no approach to this problem has been developed and intracellular stability of antibodies remains essentially unpredictable.

Summary of the Invention

Most immunoglobulins selected from phage display libraries, or otherwise, do not bind to their targets in the cytoplasm of cells. The invention concerns the use of a selection step after the *in vitro* stage to subdivide the *in vitro* binders into those which can also bind *in vivo*. Accordingly, a general immunoglobulin-target assay system is provided, in which a positive outcome (the generation of a signal) depends only on the interaction of antibody with target. This can be accomplished for many antibodies expressed in yeast and in mammalian cells.

In accordance with the present invention, therefore, there is provided a method for determining the ability of an immunoglobulin to bind to a target in an intracellular environment, comprising the steps of:

a) providing a first molecule and a second molecule, wherein stable interaction of the first and second molecules leads to the generation of a signal;

b) providing an intracellular immunoglobulin which is associated with the first molecule;

c) providing an intracellular target which is associated with the second molecule, such that association of the immunoglobulin and the antigen leads to stable interaction of the first and second molecules and generation of the signal;

d) assessing the intracellular interaction between the immunoglobulin and the target by monitoring the signal.

The basis of the method of the present invention is that when the first and second molecules are brought into stable interaction by binding of immunoglobulin to target in the intracellular environment, a signal is generated. The first and second molecules are thus two parts of a signal-generating agent which are capable of generating a signal by

interacting. A "signal", as referred to herein, is any detectable event. This may include a luminescent, fluorescent or other signal which involves the modulation of the intensity or frequency of emission or absorption of radiation; for example, a FRET signal or the induction of a luciferase gene; these and other signals are further described below.

"Intracellular" means inside a cell, and the present invention is directed to the selection of immunoglobulins which will bind to targets selectively within a cell. The cell may be any cell, prokaryotic or eukaryotic, and is preferably selected from the group consisting of a bacterial cell, a yeast cell and a higher eukaryote cell. Most preferred are yeast cells and mammalian cells. In general, the assay of the invention is carried out in the cytoplasm of the cell, and determines the ability of the immunoglobulin to fold effectively within the cytoplasm and bind to its target. As used herein, therefore, "intracellular" immunoglobulins and targets are immunoglobulins and targets which are present within a cell, preferably in the cytoplasm.

In a further embodiment, the method of the invention may be conducted under conditions which resemble or mimic an intracellular environment. Thus, "intracellular" may refer to an environment which is not within the cell, but is *in vitro*. For example, the method of the invention may be performed in an *in vitro* transcription and/or translation system, which may be obtained commercially, or derived from natural systems. Preferably, the environment is adjusted such that the reducing conditions present in cellular cytoplasm are replicated, allowing for faithful selection of immunoglobulins capable of selective binding to targets in true intracellular conditions.

Advantageously, the method of the invention further comprises a functional assay for the immunoglobulin. Thus, the method preferable further includes the step of selecting the antibodies which cause a signal to be generated in the intracellular environment, and subjecting those antibodies to a functional intracellular assay. For example, where the assay is intended to select antibodies which bind to targets which are associated with tumourigenesis, such as the gene product of the BCR-ABL chromosomal translocation, the antibodies may be tested in a cell transformation assay to determine any modulating activity on the production of transformed cells.

The first and second molecules may be any molecules, consistent with the requirement to generate a signal. They need not necessarily be polypeptides. For example, they may be fluorophores or other chemical groups capable of emitting or absorbing radiation. In a preferred aspect, however, the first and second molecules of the invention are polypeptides.

Polypeptides according to the invention associate to form a reporter molecule which is itself capable of giving a signal. Preferably, therefore, the polypeptides are domains of such a reporter molecule.

For example, the polypeptides may be domains of a fluorescent polypeptide, such as GFP, or domains of a transcription factor which, when active, upregulates transcription from a reporter gene. The reporter gene may itself encode GFP, or another detectable molecule such as luciferase, β-galactosidase, chloramphenicol acetyl transferase (CAT), an enzyme capable of catalysing an enzymatic reaction with a detectable end-point, or a molecule capable of regulating cell growth, such as by providing a required nutrient.

Association of the immunoglobulin and the target in accordance with the invention provides a stable link between the first and second molecules, which brings the molecules into stable interaction. "Stable interaction" may be defined as an interaction which permits functional cooperation of the first and second molecules in order to give rise to a detectable result, according to the signalling methods selected for use. Advantageously, a stable interaction between the first and second molecules does not occur unless the molecules are brought together through binding of the immunoglobulin and the target.

The terms "immunoglobulin" and "target" are used according to their ordinary signification given in the art, as further defined below. The term "immunoglobulin", in particular, refers to any member of the immunoglobulin superfamily, including T-cell receptors and antibodies, and includes any fragment of a natural immunoglobulin which is capable of binding to a target molecule, for example antibody fragments such as Fv and

scFv. The term "target" includes antigens, which may be targets for antibodies, T-cell receptors, or other immunoglobulin.

Preferably, the immunoglobulin is an antibody and the target is an antigen. "Antibody" explicitly includes antibody fragments.

In a preferred embodiment, the immunoglobulin and target are provided by expressing nucleic acids within the cell in which the intracellular assay is to take place. The immunoglobulin and target constructs, which comprise the signal-generating molecules, are transcribed and/or translated from nucleic acid and localised to, for instance, the cytoplasm of the cell, where the intracellular assay may take place. In other advantageous embodiments the intrcellular antibodies may be localised to any desired subcellular compartment, such as the nucleus (for example by fusion to a nuclear localisation signal), to the ER, using an ER retention signal, or other locations.

Nucleic acids encoding immunoglobulins may be obtained from libraries encoding a multiplicity of such molecules. For example, phage display libraries of antibody molecules are known and may be used in this process. Advantageously, the library encodes a repertoire of immunoglobulin molecules. A "repertoire" refers to a set of molecules generated by random, semi-random or directed variation of one or more template molecules, at the nucleic acid level, in order to provide a multiplicity of binding specificities. Methods for generating repertoires are well characterised in the art.

Libraries may moreover be constructed from nucleic acids isolated from organisms which have been challenged with an antigen. Antigen challenge will normally result in the generation of a polyclonal population of immunoglobulins, each of which is capable of binding to the antigen but which may differ from the others in terms of epitope specificity or other features. By cloning antibody genes from an organism a polyclonal population of immunoglobulins may be subjected to selection using the method of the invention in order to isolate immunoglobulins which are suitable for use in intracellular environments.

The method of the invention permits the isolation of antibodies which are capable of intracellular binding activity, and/or nucleic acids encoding such antibodies, on the basis of the signal generated by the method set forth above. Accordingly, one or both of the antibody and the antigen used in the method of the invention, together with the first or second molecules, are provided in the form of nucleic acid constructs which are transcribed to produce said antibody and/or antigen together with said first or second molecules. Nucleic acid constructs may be expression vectors capable of directing expression of the nucleic acid encoding the antibody in the cell in which the method of the invention is to be performed.

The present invention allows the isolation of antibodies and/or nucleic acids encoding them which bind to antigens intracellularly. Advantageously, that antibodies which are screened by the method of the present invention are previously selected for antigen specificity. Accordingly, the invention provides a method for preparing an antibody suitable for use in a procedure according to the invention, comprising the steps of:

a) expressing a repertoire of antibody genes in a selection system and isolating those genes which encode antibodies specific for a desired antigen;

b) bringing the isolated genes into operative association with nucleic acids encoding a first molecule, wherein stable interaction of the first molecule with a second molecule generates a signal, in order to produce a fusion polypeptide comprising the antibody and the first molecule.

As used above, "operative association" refers to the fusion or juxtaposition of coding sequences such that a fusion protein is produced, comprising the antibody and the signal-generating molecule. Normally, performing a selection against an antigen will generate a smaller repertoire of antibodies which share antigen specificity. The transcription units encoding such antibodies, fused to the signal generating molecules, are employed in an assay according to the invention in order to select those antibodies which are capable of functioning intracellularly.

Brief Description of the Drawings

Figure 1.

AMCV Antigen- scFv F8 antibody interactions in the yeast two-hybrid system

L40 his- yeast cells (auxotrophic for Trp and Leu and with *HIS3* and *lacZ* reporter genes for VP16-dependent transcriptional activation) are co-transfetced with a LexA-AMCV antigen bait vector (AMCVp41/BTM116) and scFv-VP16 fusion vectors or the VP16 vector alone. Yeast clones are grown on agar and appear slightly pink (because of the ade2-101 mutation). Interaction of antigen bait and ScFv-VP16 fusion causes growth on his-minus plates due to HIS3 activation and blue colour in Xgal substrate due to *lacZ* gene activation.

β-galactosidase assays of L40 yeast transfected with the following vectors are shown:

Row 1: AMCVp41/BTM116 alone
Row 2: AMCVp41/BTM116 + VP16 vector
Row 3: Lamin/BTM116 + scFvF8/VP16
Row 4: AMCVp41/BTM116 + scFvF8/VP16

β-galactosidase activation is only observed when the LexA-AMCV bait is co-expressed with scFvF8 (The expression of LexA, LexA-AMCVp41 and LexA-Lamin protein is assessed by western blot analysis using an anti LexA polyclonal antibody).

Figure 2.

Intracellular interaction of scFv with target antigens in yeast

L40 yeast are co-transfected with LexA-antigen DNA-binding fusions (baits) in the pBTM116 vector and scFv fusions with the VP16 transcriptional transactivation domain in the pVP16 vector. Yeast are streaked and grown on his- minus medium and scored for β-galactosidase activity (yielding blue colonies when the scFv-antigen interaction occurs intracellularly).

A. Transfection of HIV-1 integrase clone HIV-1 IN/BTM116 alone (row 1), with the pVP16 vector (row 2) or with the anti-integrase scFvIN33/VP16 (row 4). In row 3 scFvIN33/VP16 is co-transfected with the non-relevant Lamin/BTM116 bait. β-gal activation is only observed in row 4.

B. Transfection of the Syk bait clone Syk/BTM116 alone (row 1) or with pVP16 (row 2). In rows 3, 4 and 5 the three anti-Syk scFv-VP16 clones (scFvV6C11, G6G2 and G4G11-VP16) are co-transfected with a non-relevant antigen bait (Lamin/BTM116) and in rows 6, 7 and 8 the same three anti-Syk scFv-VP16 clones are co-transfected with the Syk/BTM116 bait. β-gal activation is only observed in row 8.

C. Transfection of the K-ras bait clone K-ras/BTM116 alone (row 1) or with pVP16 (row 2). In row 3, the anti-ras scFv clone scFvY13/VP16 is co-transfected with the non-relevant Lamin/BTM116 bait at 20–C. Rows 4 and 5 are clones co-transfected with K-ras/BTM116 and scFvY13/VP16 grown at 30–C and 20–C respectively. β-gal activation is only observed in row 5.

Figure 3.

Plating assay for histidine prototrophy

L40 yeast are transfected bait clones alone (A), with baits together with the pVP16 vector (B) and assayed for growth on the indicated selective media. Only β-gal/BTM116 bait gave a background of histidine prototrophs when expressed alone (panel A) or together with pVP16 (panel B). All of the scFv-VP16 fragments tested interact specifically with their specific bait but not with a non-relevant antigen (Lamin) (panel C).

(+) indicates growth and (-) no growth.

YC-WHULK indicates yeast colonies grown in the absence of trp, his, ura, leu and lys; YC-WHUK in the absence of trp, his, ura and lys; YC-WL in the absence of trp and leu and YC-W in the absence of trp.

Figure 4.

Mammalian antibody-antigen two-hybrid CAT assay

A chloramphenicol acetyl transferase CAT reporter clone (pE5C-CAT) is transfected into CHO cells with combinations of pM1 or pNLVP16 derivatives and CAT activity scored by thin-layer chromatography (panel A) or phosphoimager analysis (panel B). Two independent CAT assays are performed for each transfection point.

Each transfection has pE5C-CAT reporter together with Lane 1: pM-βgal + pNLscFvR4-VP16; Lane 2: pE5C-CAT reporter alone; Lane 3: pM-β-gal; Lane 4: pNLscFvR4-VP16; Lane 5: pMβ–gal + pNLscFvF8-VP16; Lane 6: pNLscFv-VP16 + pM1-AMCV; Lane 7: pM-scFvR4 + pNLVP16-βgal; Lane 8: pM-scFvR4; Lane 9: pNLVP16-βgal; Lane 10: pM-βgal + pNLVP16-scFvR4; Lane 11: pNLVP16-scFvR4

Figure 5.

Redox state of scFv fragments

Western blot analysis of scFvF8-VP16 (expressed in the cytoplasm of L40 yeast cells) and of scFvαD11 (expressed as a soluble protein in Baculovirus expression system). Samples are prepared as described in the Examples, and separated in the presence (reducing) or absence (non-reducing) of β-mercaptoethanol (β-mer) in SDS-PAGE loading buffer. After blotting, the scFvs are detected with the anti-myc antibody 9E10. The bars at right of the lanes indicate the molecular weight gel shift between the oxidised (ox) and reduced (red) forms of the scFvαD11. ScFvF8-VP16 fusion protein does not undergo any difference in electrophoretic mobility between the sample analysed under reducing and non-reducing conditions, indicating that scFvF8-VP16 does not form disulphide bonds in yeast cytoplasm (RED).

Figure 6.

Model selection of scFv-VP16 clone from a mixture of scFv

Yeast L40 is co-transformed with 3 different DNA mixtures, with 3 increasing dilutions of scFvF8-VP16 DNA progressively diluted with DNA encoding non relevant scFv library.

Top plates: co-transformed cells are plated onto His- plates and after 3 days, growing colonies are picked and replated on grid His- plates. After 3 days growth, a β-galactosidase assay is performed. All the surviving colonies revealed a positive interaction.

Bottom plates: co-transformed cells are plated onto His+ plates and after 3 days, growing colonies are picked and replated on grid His+ plates. After 3 days, colonies are screened for β-gal activity. As expected not all the colonies are blue indicating that it is possible to detect interacting Ag-scFv fragments pairs against a background of non-relevant scFv fragments, even when selecting the co-transformed cells only for the presence of plasmids.

YC-WHULK indicates yeast colonies grown in the absence of trp, his, ura, leu and lys and YC-WL in the absence of trp and leu.

Detailed Description of the Invention

a) Immunoglobulins

Immunoglobulin molecules, according to the present invention, are in the broadest sense members of the immunoglobulin superfamily, a family of polypeptides which comprise the immunoglobulin fold characteristic of antibody molecules, which contains two β sheets and, usually, a conserved disulphide bond. Members of the immunoglobulin superfamily are involved in many aspects of cellular and non-cellular interactions *in vivo*, including widespread roles in the immune system (for example, antibodies, T-cell receptor molecules and the like), involvement in cell adhesion (for example the ICAM molecules) and intracellular signalling (for example, receptor molecules, such as the PDGF receptor). The present invention is applicable to all immunoglobulin superfamily

molecules which are capable of binding to target molecules. Preferably, the present invention relates to antibodies.

Antibodies, as used herein, refers to complete antibodies or antibody fragments capable of binding to a selected target, and including Fv, ScFv, Fab' and $F(ab')_2$, monoclonal and polyclonal antibodies, engineered antibodies including chimeric, CDR-grafted and humanised antibodies, and artificially selected antibodies produced using phage display or alternative techniques. Small fragments, such as Fv and ScFv, possess advantageous properties for diagnostic and therapeutic applications on account of their small size and consequent superior tissue distribution. Preferably, the antibody is a single chain antibody or scFv.

The antibodies according to the invention are especially indicated for diagnostic and therapeutic applications. Accordingly, they may be altered antibodies comprising an effector protein such as a toxin or a label. Especially preferred are labels which allow the imaging of the distribution of the antibody in vivo. Such labels may be radioactive labels or radioopaque labels, such as metal particles, which are readily visualisable within the body of a patient. Moreover, they may be fluorescent labels or other labels which are visualisable on tissue samples removed from patients. Effector groups may be added prior to the selection of the antibodies by the method of the present invention, or afterwards.

Antibodies may be obtained from animal serum, or, in the case of monoclonal antibodies or fragments thereof, produced in cell culture. Recombinant DNA technology may be used to produce the antibodies according to established procedure, in bacterial or preferably mammalian cell culture. The selected cell culture system preferably secretes the antibody product.

Multiplication of hybridoma cells or mammalian host cells in vitro is carried out in suitable culture media, which are the customary standard culture media, for example Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium, optionally replenished by a mammalian serum, e.g. foetal calf serum, or trace elements and growth

sustaining supplements, e.g. feeder cells such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages, 2-aminoethanol, insulin, transferrin, low density lipoprotein, oleic acid, or the like. Multiplication of host cells which are bacterial cells or yeast cells is likewise carried out in suitable culture media known in the art, for example for bacteria in medium LB, NZCYM, NZYM, NZM, Terrific Broth, SOB, SOC, 2 x YT, or M9 Minimal Medium, and for yeast in medium YPD, YEPD, Minimal Medium, or Complete Minimal Dropout Medium.

In vitro production provides relatively pure antibody preparations and allows scale-up to give large amounts of the desired antibodies. Techniques for bacterial cell, yeast or mammalian cell cultivation are known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or immobilised or entrapped cell culture, e.g. in hollow fibres, microcapsules, on agarose microbeads or ceramic cartridges.

Large quantities of the desired antibodies can also be obtained by multiplying mammalian cells in vivo. For this purpose, hybridoma cells producing the desired antibodies are injected into histocompatible mammals to cause growth of antibody-producing tumours. Optionally, the animals are primed with a hydrocarbon, especially mineral oils such as pristane (tetramethyl-pentadecane), prior to the injection. After one to three weeks, the antibodies are isolated from the body fluids of those mammals. For example, hybridoma cells obtained by fusion of suitable myeloma cells with antibody-producing spleen cells from Balb/c mice, or transfected cells derived from hybridoma cell line Sp2/0 that produce the desired antibodies are injected intraperitoneally into Balb/c mice optionally pre-treated with pristane, and, after one to two weeks, ascitic fluid is taken from the animals.

The foregoing, and other, techniques are discussed in, for example, Kohler and Milstein, (1975) Nature 256:495-497; US 4,376,110; Harlow and Lane, Antibodies: a Laboratory Manual, (1988) Cold Spring Harbor, incorporated herein by reference. Techniques for the preparation of recombinant antibody molecules is described in the above references

and also in, for example, EP 0623679; EP 0368684 and EP 0436597, which are incorporated herein by reference.

The cell culture supernatants are screened for the desired antibodies, preferentially by immunofluorescent staining of cells expressing the desired target by immunoblotting, by an enzyme immunoassay, e.g. a sandwich assay or a dot-assay, or a radioimmunoassay.

For isolation of the antibodies, the immunoglobulins in the culture supernatants or in the ascitic fluid may be concentrated, e.g. by precipitation with ammonium sulphate, dialysis against hygroscopic material such as polyethylene glycol, filtration through selective membranes, or the like. If necessary and/or desired, the antibodies are purified by the customary chromatography methods, for example gel filtration, ion-exchange chromatography, chromatography over DEAE-cellulose and/or (immuno-)affinity chromatography, e.g. affinity chromatography with the target molecule or with Protein-A.

Antibodies generated according to the foregoing procedures may be cloned by isolation of nucleic acid from cells, according to standard procedures. Usefully, nucleic acids variable domains of the antibodies may be isolated and used to construct antibody fragments, such as scFv.

The invention therefore preferably employs recombinant nucleic acids comprising an insert coding for a heavy chain variable domain and/or for a light chain variable domain of antibodies. By definition such nucleic acids comprise coding single stranded nucleic acids, double stranded nucleic acids consisting of said coding nucleic acids and of complementary nucleic acids thereto, or these complementary (single stranded) nucleic acids themselves.

Furthermore, nucleic acids encoding a heavy chain variable domain and/or for a light chain variable domain of antibodies can be enzymatically or chemically synthesised nucleic acids having the authentic sequence coding for a naturally-occurring heavy chain variable domain and/or for the light chain variable domain, or a mutant thereof. A mutant of the authentic sequence is a nucleic acid encoding a heavy chain variable domain and/or

a light chain variable domain of the above-mentioned antibodies in which one or more amino acids are deleted or exchanged with one or more other amino acids. Preferably said modification(s) are outside the CDRs of the heavy chain variable domain and/or of the light chain variable domain of the antibody. Such a mutant nucleic acid is also intended to be a silent mutant wherein one or more nucleotides are replaced by other nucleotides with the new codons coding for the same amino acid(s). Such a mutant sequence is also a degenerated sequence. Degenerated sequences are degenerated within the meaning of the genetic code in that an unlimited number of nucleotides are replaced by other nucleotides without resulting in a change of the amino acid sequence originally encoded. Such degenerated sequences may be useful due to their different restriction sites and/or frequency of particular codons which are preferred by the specific host, particularly yeast, bacterial or mammalian cells, to obtain an optimal expression of the heavy chain variable domain and/or a light chain variable domain.

The term mutant is intended to include a DNA mutant obtained by *in vitro* or *in vivo* mutagenesis of DNA according to methods known in the art.

Recombinant DNA technology may be used to improve the antibodies of the invention. Thus, chimeric antibodies may be constructed in order to decrease the immunogenicity thereof in diagnostic or therapeutic applications. Moreover, immunogenicity may be minimised by humanising the antibodies by CDR grafting [see European Patent Application 0 239 400 (Winter)] and, optionally, framework modification [see international patent application WO 90/07861 (Protein Design Labs)].

The invention therefore also employs recombinant nucleic acids comprising an insert coding for a heavy chain variable domain of an antibody fused to a human constant domain γ, for example γ1, γ2, γ3 or γ4, preferably γ1 or γ4. Likewise the invention concerns recombinant DNAs comprising an insert coding for a light chain variable domain of an antibody fused to a human constant domain κ or λ, preferably κ.

More preferably, the invention employs CDR-grafted antibodies, which are preferable CDR-grafted light chain and heavy chain variable domains only. Advantageously, the

heavy chain variable domain and the light chain variable domain are linked by way of a spacer group, optionally comprising a signal sequence facilitating the processing of the antibody in the host cell and/or a DNA coding for a peptide facilitating the purification of the antibody and/or a cleavage site and/or a peptide spacer and/or an effector molecule. Such antibodies are known as scFvs.

Antibodies may moreover be generated by mutagenesis of antibody genes to produce artificial repertoires of antibodies. This technique allows the preparation of antibody libraries, as discussed further below; antibody libraries are also available commercially. Hence, the present invention advantageously employs artificial repertoires of immunoglobulins, preferably artificial ScFv repertoires, as an immunoglobulin source.

b) Targets

Targets are chosen according to the use to which it is intended to put the intracellular immunoglobulin selected by the method of the present invention. Thus, where it is desired to select an immunoglobulin capable of binding to a defined cellular component, such as a polypeptide, a subcellular structure or an intracellular pathogen, the whole of said component or an epitope derived therefrom may be used as a target.

Potential targets include polypeptides, particularly nascent polypeptides or intracellular polypeptide precursors, which are present in the cell. Advantageously, the target is a mutant polypeptide, such as a polypeptide generated through genetic mutation, including point mutations, deletions and chromosomal translocations. Such polypeptides are frequently involved in tumourigenesis. Examples include the gene product produced by the spliced BCR-ABL genes and point mutants of the *Ras* oncogene. The invention is moreover applicable to all mutated oncogene products, all chromosomal translocated oncogene products (especially fusion proteins), aberrant proteins in expressed in disease, and viral or bacerial specific proteins expressed as a result of infection.

The target may alternatively be an RNA molecule, for example a precursor RNA or a mutant RNA species generated by genetic mutation or otherwise.

The target may be inserted into the cell, for example as described below, or may be endogenous to the cell. Where the target is endogenous, generation of the signal is dependent on the attachment of a signalling molecule to the target within the cell, or on the target itself being capable of functioning as one half of the signal-generating agent.

c) Libraries and selection systems

Immunoglobulins for use in the invention may be isolated from libraries comprising artificial repertoires of immunoglobulin polypeptides. Advantageously, the immunoglobulins may be preselected by screening against the desired target, such that the method of the invention is performed with immunoglobulins which substantially all are specific for the intended target.

Any library selection system may be used in conjunction with the invention. Selection protocols for isolating desired members of large libraries are known in the art, as typified by phage display techniques. Such systems, in which diverse peptide sequences are displayed on the surface of filamentous bacteriophage (Scott and Smith (1990 *supra*), have proven useful for creating libraries of antibody fragments (and the nucleotide sequences that encoding them) for the *in vitro* selection and amplification of specific antibody fragments that bind a target antigen. The nucleotide sequences encoding the V_H and V_L regions are linked to gene fragments which encode leader signals that direct them to the periplasmic space of *E. coli* and as a result the resultant antibody fragments are displayed on the surface of the bacteriophage, typically as fusions to bacteriophage coat proteins (e.g., pIII or pVIII). Alternatively, antibody fragments are displayed externally on lambda phage capsids (phagebodies). An advantage of phage-based display systems is that, because they are biological systems, selected library members can be amplified simply by growing the phage containing the selected library member in bacterial cells. Furthermore, since the nucleotide sequence that encode the polypeptide library member is contained on a phage or phagemid vector, sequencing, expression and subsequent genetic manipulation is relatively straightforward.

Methods for the construction of bacteriophage antibody display libraries and lambda phage expression libraries are well known in the art (McCafferty *et al.* (1990) **supra**; Kang *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.*, **88**: 4363; Clackson *et al.* (1991) *Nature*, **352**: 624; Lowman *et al.* (1991) *Biochemistry*, **30**: 10832; Burton *et al.* (1991) *Proc. Natl. Acad. Sci U.S.A.*, **88**: 10134; Hoogenboom *et al.* (1991) Nucleic Acids Res., **19**: 4133; Chang *et al.* (1991) *J. Immunol.*, **147**: 3610; Breitling *et al.* (1991) *Gene*, **104**: 147; Marks *et al.* (1991) supra; Barbas *et al.* (1992) supra; Hawkins and Winter (1992) *J. Immunol.*, **22**: 867; Marks *et al.*, 1992, *J. Biol. Chem.*, **267**: 16007; Lerner *et al.* (1992) *Science*, **258**: 1313, incorporated herein by reference).

One particularly advantageous approach has been the use of scFv phage-libraries (Huston *et al.*, 1988, Proc. Natl. Acad. Sci U.S.A., 85: 5879-5883; Chaudhary *et al.* (1990) Proc. Natl. Acad. Sci U.S.A., 87: 1066-1070; McCafferty *et al.* (1990) supra; Clackson *et al.* (1991) supra; Marks *et al.* (1991) supra; Chiswell *et al.* (1992) Trends Biotech., **10**: 80; Marks *et al.* (1992) supra). Various embodiments of scFv libraries displayed on bacteriophage coat proteins have been described. Refinements of phage display approaches are also known, for example as described in WO96/06213 and WO92/01047 (Medical Research Council *et al.*) and WO97/08320 (Morphosys, supra), which are incorporated herein by reference.

Alternative library selection technologies include bacteriophage lambda expression systems, which may be screened directly as bacteriophage plaques or as colonies of lysogens, both as previously described (Huse *et al.* (1989) *Science*, **246**: 1275; Caton and Koprowski (1990) *Proc. Natl. Acad. Sci. U.S.A.*, **87**; Mullinax *et al.* (1990) *Proc. Natl. Acad. Sci. U.S.A.*, **87**: 8095; Persson *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.*, **88**: 2432) and are of use in the invention. Whilst such expression systems can be used to screening up to 10^6 different members of a library, they are not really suited to screening of larger numbers (greater than 10^6 members). Other screening systems rely, for example, on direct chemical synthesis of library members. One early method involves the synthesis of peptides on a set of pins or rods, such as described in WO84/03564. A similar method involving peptide synthesis on beads, which forms a peptide library in which each bead is an individual library member, is described in U.S. Patent No. 4,631,211 and a related

method is described in WO92/00091. A significant improvement of the bead-based methods involves tagging each bead with a unique identifier tag, such as an oligonucleotide, so as to facilitate identification of the amino acid sequence of each library member. These improved bead-based methods are described in WO93/06121.

Another chemical synthesis method involves the synthesis of arrays of peptides (or peptidomimetics) on a surface in a manner that places each distinct library member (e.g., unique peptide sequence) at a discrete, predefined location in the array. The identity of each library member is determined by its spatial location in the array. The locations in the array where binding interactions between a predetermined molecule (e.g., a receptor) and reactive library members occur is determined, thereby identifying the sequences of the reactive library members on the basis of spatial location. These methods are described in U.S. Patent No. 5,143,854; WO90/15070 and WO92/10092; Fodor *et al.* (1991) *Science*, **251**: 767; Dower and Fodor (1991) *Ann. Rep. Med. Chem.*, **26**: 271.

Other systems for generating libraries of polypeptides or nucleotides involve the use of cell-free enzymatic machinery for the *in vitro* synthesis of the library members. In one method, RNA molecules are selected by alternate rounds of selection against a target ligand and PCR amplification (Tuerk and Gold (1990) *Science*, **249**: 505; Ellington and Szostak (1990) *Nature*, **346**: 818). A similar technique may be used to identify DNA sequences which bind a predetermined human transcription factor (Thiesen and Bach (1990) *Nucleic Acids Res.*, **18**: 3203; Beaudry and Joyce (1992) *Science*, **257**: 635; WO92/05258 and WO92/14843). In a similar way, *in vitro* translation can be used to synthesise polypeptides as a method for generating large libraries. These methods which generally comprise stabilised polysome complexes, are described further in WO88/08453, WO90/05785, WO90/07003, WO91/02076, WO91/05058, and WO92/02536. Alternative display systems which are not phage-based, such as those disclosed in WO95/22625 and WO95/11922 (Affymax) use the polysomes to display polypeptides for selection. These and all the foregoing documents also are incorporated herein by reference.

An alternative to the use of phage or other cloned libraries is to use nucleic acid, preferably RNA, derived from the spleen of an animal which has been immunised with

the selected target. RNA thus obtained represents a natural library of immunoglobulins. Isolation of V-region and C-region mRNA permits antibody fragments, such as Fab or Fv, to be expressed intracellularly in accordance with the invention.

Briefly, RNA is isolated from the spleen of an immunised animal and PCR primers used to amplify V_H and V_L cDNA selectively from the RNA pool. The V_H and V_L sequences thus obtained are joined to make scFv antibodies. PCR primer sequences are based on published V_H and V_L sequences and are available commercially in kit form.

d) Delivery of Immunoglobulins and Targets to Cells

The present invention provides an assay for intracellular antibodies which is conducted essentially intracellularly, or in conditions which mimic the intracellular environment, preferably the cytoplasmic environment.

In order to introduce immunoglobulins and target molecules into an intracellular environment, cells are advantageously transfected with nucleic acids which encode the immunoglobulins and/or their targets.

Nucleic acids encoding immunoglobulins and/or targets can be incorporated into vectors for expression. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for expression thereof. Selection and use of such vehicles are well within the skill of the artisan. Many vectors are available, and selection of appropriate vector will depend on the intended use of the vector, the size of the nucleic acid to be inserted into the vector, and the host cell to be transformed with the vector. Each vector contains various components depending on its function and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: an origin of replication, one or more marker genes, an enhancer element, a promoter, a transcription termination sequence and a signal sequence.

Moreover, nucleic acids encoding the immunoglobulins and/or targets according to the invention may be incorporated into cloning vectors, for general manipulation and nucleic acid amplification purposes.

Both expression and cloning vectors generally contain nucleic acid sequence that enable the vector to replicate in one or more selected host cells. Typically in cloning vectors, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2m plasmid origin is suitable for yeast, and various viral origins (e.g. SV 40, polyoma, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors unless these are used in mammalian cells competent for high level DNA replication, such as COS cells.

Most expression vectors are shuttle vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another class of organisms for expression. For example, a vector is cloned in E. coli and then the same vector is transfected into yeast or mammalian cells even though it is not capable of replicating independently of the host cell chromosome. DNA may also be replicated by insertion into the host genome. However, the recovery of genomic DNA is more complex than that of exogenously replicated vector because restriction enzyme digestion is required to excise the nucleic acid. DNA can be amplified by PCR and be directly transfected into the host cells without any replication component.

Advantageously, an expression and cloning vector may contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and

other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available from complex media.

As to a selective gene marker appropriate for yeast, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker gene. Suitable markers for yeast are, for example, those conferring resistance to antibiotics G418, hygromycin or bleomycin, or provide for prototrophy in an auxotrophic yeast mutant, for example the URA3, LEU2, LYS2, TRP1, or HIS3 gene.

Since the replication of vectors is conveniently done in *E. coli*, an *E. coli* genetic marker and an E. coli origin of replication are advantageously included. These can be obtained from E. coli plasmids, such as pBR322, Bluescript© vector or a pUC plasmid, e.g. pUC18 or pUC19, which contain both an *E. coli* replication origin and an *E. coli* genetic marker conferring resistance to antibiotics, such as ampicillin.

Suitable selectable markers for mammalian cells are those that enable the identification of cells expressing the desired nucleic acid, such as dihydrofolate reductase (DHFR, methotrexate resistance), thymidine kinase, or genes conferring resistance to G418 or hygromycin. The mammalian cell transformants are placed under selection pressure which only those transformants which have taken up and are expressing the marker are uniquely adapted to survive. In the case of a DHFR or glutamine synthase (GS) marker, selection pressure can be imposed by culturing the transformants under conditions in which the pressure is progressively increased, thereby leading to amplification (at its chromosomal integration site) of both the selection gene and the linked nucleic acid. Amplification is the process by which genes in greater demand for the production of a protein critical for growth, together with closely associated genes which may encode a desired protein, are reiterated in tandem within the chromosomes of recombinant cells. Increased quantities of desired protein are usually synthesised from thus amplified DNA.

Expression and cloning vectors usually contain a promoter that is recognised by the host organism and is operably linked to the desired nucleic acid. Such a promoter may be inducible or constitutive. The promoters are operably linked to the nucleic acid by

removing the promoter from the source DNA and inserting the isolated promoter sequence into the vector. Both the native promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of nucleic acid encoding the immunoglobulin or target molecule. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

Promoters suitable for use with prokaryotic hosts include, for example, the β-lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (trp) promoter system and hybrid promoters such as the tac promoter. Their nucleotide sequences have been published, thereby enabling the skilled worker operably to ligate them a desired nucleic acid, using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems will also generally contain a Shine-Delgarno sequence operably linked to the nucleic acid.

Preferred expression vectors are bacterial expression vectors which comprise a promoter of a bacteriophage such as phagex or T7 which is capable of functioning in the bacteria. In one of the most widely used expression systems, the nucleic acid encoding the fusion protein may be transcribed from the vector by T7 RNA polymerase (Studier et al, Methods in Enzymol. 185; 60-89, 1990). In the *E. coli* BL21(DE3) host strain, used in conjunction with pET vectors, the T7 RNA polymerase is produced from the λ-lysogen DE3 in the host bacterium, and its expression is under the control of the IPTG inducible lac UV5 promoter. This system has been employed successfully for over-production of many proteins. Alternatively the polymerase gene may be introduced on a lambda phage by infection with an int- phage such as the CE6 phage which is commercially available (Novagen, Madison, USA). other vectors include vectors containing the lambda PL promoter such as PLEX (Invitrogen, NL) , vectors containing the trc promoters such as pTrcHisXpressTm (Invitrogen) or pTrc99 (Pharmacia Biotech, SE) , or vectors containing the tac promoter such as pKK223-3 (Pharmacia Biotech) or PMAL (new England Biolabs, MA, USA).

Suitable promoting sequences for use with yeast hosts may be regulated or constitutive and are preferably derived from a highly expressed yeast gene, especially a Saccharomyces cerevisiae gene. Thus, the promoter of the TRP1 gene, the ADH1 or ADHII gene, the acid phosphatase (PH05) gene, a promoter of the yeast mating pheromone genes coding for the a- or α-factor or a promoter derived from a gene encoding a glycolytic enzyme such as the promoter of the enolase, glyceraldehyde-3-phosphate dehydrogenase (GAP), 3-phospho glycerate kinase (PGK), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triose phosphate isomerase, phosphoglucose isomerase or glucokinase genes, the *S. cerevisiae* GAL 4 gene, the *S. pombe* nmt 1 gene or a promoter from the TATA binding protein (TBP) gene can be used. Furthermore, it is possible to use hybrid promoters comprising upstream activation sequences (UAS) of one yeast gene and downstream promoter elements including a functional TATA box of another yeast gene, for example a hybrid promoter including the UAS(s) of the yeast PH05 gene and downstream promoter elements including a functional TATA box of the yeast GAP gene (PH05-GAP hybrid promoter). A suitable constitutive PHO5 promoter is e.g. a shortened acid phosphatase PH05 promoter devoid of the upstream regulatory elements (UAS) such as the PH05 (-173) promoter element starting at nucleotide -173 and ending at nucleotide -9 of the PH05 gene.

Gene transcription from vectors in mammalian hosts may be controlled by promoters derived from the genomes of viruses such as polyoma virus, adenovirus, fowlpox virus, bovine papilloma virus, avian sarcoma virus, cytomegalovirus (CMV), a retrovirus and Simian Virus 40 (SV40), from heterologous mammalian promoters such as the actin promoter or a very strong promoter, e.g. a ribosomal protein promoter, and from promoters normally associated with immunoglobulin sequences.

Transcription of a nucleic acid by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are relatively orientation and position independent. Many enhancer sequences are known from mammalian genes (e.g. elastase and globin). However, typically one will employ an enhancer from a eukaryotic cell

virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270) and the CMV early promoter enhancer. The enhancer may be spliced into the vector at a position 5' or 3' to the desired nucleic acid, but is preferably located at a site 5' from the promoter.

Advantageously, a eukaryotic expression vector may comprise a locus control region (LCR). LCRs are capable of directing high-level integration site independent expression of transgenes integrated into host cell chromatin, which is of importance especially where the gene is to be expressed in the context of a permanently-transfected eukaryotic cell line in which chromosomal integration of the vector has occurred.

Eukaryotic expression vectors will also contain sequences necessary for the termination of transcription and for stabilising the mRNA. Such sequences are commonly available from the 5' and 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the immunoglobulin or the target.

Particularly useful for practising the present invention are expression vectors that provide for the transient expression of nucleic acids in mammalian cells. Transient expression usually involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector, and, in turn, synthesises high levels of the desired gene product.

Construction of vectors according to the invention may employ conventional ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required. If desired, analysis to confirm correct sequences in the constructed plasmids is performed in a known fashion. Suitable methods for constructing expression vectors, preparing in vitro transcripts, introducing DNA into host cells, and performing analyses for assessing gene product expression and function are known to those skilled in the art. Gene presence, amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA, dot blotting (DNA or RNA

analysis), or in situ hybridisation, using an appropriately labelled probe which may be based on a sequence provided herein. Those skilled in the art will readily envisage how these methods may be modified, if desired.

Immunoglobulins and/or targets may be directly introduced to the cell by microinjection, or delivery using vesicles such as liposomes which are capable of fusing with the cell membrane. Viral fusogenic peptides are advantageously used to promote membrane fusion and delivery to the cytoplasm of the cell.

e) Generation of a Signal

In the method of the present invention, a signal is advantageously generated by the interaction of two molecules, brought together by the binding of the immunoglobulin to the target. The signal generated will thus be dependent on the nature of the molecules used in the method of the invention.

In a first embodiment, the signal-generation molecules may be fluorophores. Particularly preferred are fluorescent molecules which participate in energy transfer (FRET).

FRET is detectable when two fluorescent labels which fluoresce at different frequencies are sufficiently close to each other that energy is able to be transferred from one label to the other. FRET is widely known in the art (for a review, see Matyus, 1992, J. Photochem. Photobiol. B: Biol., 12: 323-337, which is herein incorporated by reference). FRET is a radiationless process in which energy is transferred from an excited donor molecule to an acceptor molecule; the efficiency of this transfer is dependent upon the distance between the donor an acceptor molecules, as described below. Since the rate of energy transfer is inversely proportional to the sixth power of the distance between the donor and acceptor, the energy transfer efficiency is extremely sensitive to distance changes. Energy transfer is said to occur with detectable efficiency in the 1-10 nm distance range, but is typically 4-6 nm for favourable pairs of donor and acceptor.

Radiationless energy transfer is based on the biophysical properties of fluorophores. These principles are reviewed elsewhere (Lakowicz, 1983, Principles of Fluorescence Spectroscopy, Plenum Press, New York; Jovin and Jovin, 1989, Cell Structure and Function by Microspectrofluorometry, eds. E. Kohen and J.G. Hirschberg, Academic Press, both of which are incorporated herein by reference). Briefly, a fluorophore absorbs light energy at a characteristic wavelength. This wavelength is also known as the excitation wavelength. The energy absorbed by a fluorochrome is subsequently released through various pathways, one being emission of photons to produce fluorescence. The wavelength of light being emitted is known as the emission wavelength and is an inherent characteristic of a particular fluorophore. Radiationless energy transfer is the quantum-mechanical process by which the energy of the excited state of one fluorophore is transferred without actual photon emission to a second fluorophore. That energy may then be subsequently released at the emission wavelength of the second fluorophore. The first fluorophore is generally termed the donor (D) and has an excited state of higher energy than that of the second fluorophore, termed the acceptor (A). The essential features of the process are that the emission spectrum of the donor overlap with the excitation spectrum of the acceptor, and that the donor and acceptor be sufficiently close. The distance over which radiationless energy transfer is effective depends on many factors including the fluorescence quantum efficiency of the donor, the extinction coefficient of the acceptor, the degree of overlap of their respective spectra, the refractive index of the medium, and the relative orientation of the transition moments of the two fluorophores. In addition to having an optimum emission range overlapping the excitation wavelength of the other fluorophore, the distance between D and A must be sufficiently small to allow the radiationless transfer of energy between the fluorophores.

In a FRET assay, the fluorescent molecules are chosen such that the excitation spectrum of one of the molecules (the acceptor molecule) overlaps with the emission spectrum of the excited fluorescent molecule (the donor molecule). The donor molecule is excited by light of appropriate intensity within the donor's excitation spectrum. The donor then emits some of the absorbed energy as fluorescent light and dissipates some of the energy by FRET to the acceptor fluorescent molecule. The fluorescent energy it produces is quenched by the acceptor fluorescent molecule. FRET can be manifested as a reduction

in the intensity of the fluorescent signal from the donor, reduction in the lifetime of its excited state, and re-emission of fluorescent light at the longer wavelengths (lower energies) characteristic of the acceptor. When the donor and acceptor molecules become spatially separated, FRET is diminished or eliminated.

Suitable fluorophores are known in the art, and include chemical fluorophores and fluorescent polypeptides, such as GFP and mutants thereof which fluoresce with different wavelengths or intensities (see WO 97/28261). Chemical fluorophores may be attached to immunoglobulin or target molecules by incorporating binding sites therefor into the immunoglobulin or target molecule during the synthesis thereof.

Preferably, however, the fluorophore is a fluorescent protein, which is advantageously GFP or a mutant thereof. GFP and its mutants may be synthesised together with the immunoglobulin or target molecule by expression therewith as a fusion polypeptide, according to methods well known in the art. For example, a transcription unit may be constructed as an in-frame fusion of the desired GFP and the immunoglobulin or target, and inserted into a vector as described above, using conventional PCR cloning and ligation techniques.

In a second embodiment, the immunoglobulin and target polypeptides are associated with molecules which give rise to a biological signal. Preferred are polypeptide molecules, which advantageously interact to form a transcription factor, or another regulatory molecule, which modulates gene expression within the cell.

Exemplary transcription factor molecules have been described in the literature, for example by Fields & Song, (1989) Nature 340:245-246, which is incorporated herein by reference. In a preferred embodiment, the immunoglobulin molecule is expressed as fusion protein with the activation domain of the HSV1 VP16 molecule. This transcription factor domain is capable of upregulating gene transcription from a promoter to which it is bound through a DNA binding activity. The latter is provided by the DNA-binding domain of the *E. coli* LexA polypeptide, which is expressed as a fusion protein with the target polypeptide.

The biological signal may be any detectable signal, such as the induction of the expression of a detectable gene product. Examples of detectable gene products include bioluminescent polypeptides, such as luciferase and GFP, polypeptides detectable by specific assays, such as β-galactosidase and CAT, and polypeptides which modulate the growth characteristics of the host cell, such as enzymes required for metabolism such as HIS3, or antibiotic resistance genes such as G418. In a preferred aspect of the invention, the signal is detectable at the cell surface. For example, the signal may be a luminescent or fluorescent signal, which is detectable from outside the cell and allows cell sorting by FACS or other optical sorting techniques. Alternatively, the signal may comprise the expression of a cell surface marker, such as a CD molecule, for example CD4 or CD8, which may itself be labelled, for example with a fluorescent group, or may be detectable using a labelled antibody.

In this embodiment, the invention permits the screening of entire antibody libraries, such as phage libraries, without prior application of phage display to isolate the antibodies which bind to the desired antigen. Use of optical sorting, such as FACS, enables an entire library to be panned and selects for antibodies which are capable of functioning intracellularly and bind the desired target.

The invention is further described, for the purposes of illustration only, in the following examples.

Examples

Example 1

Detection of intracellular antibody binding

A powerful system used for detecting protein-protein interactions is the eukaryotic two-hybrid assay (Fields, S. & Song, O. (1989) **340**: 245-246). This assay is adapted for expression of scFv fragments and corresponding antigens in yeast, and detection of binding by the scFv to the antigens intracellularly.

In general, yeast strains are grown in rich medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose, and 0.1% mg/ml adenine buffered at pH5.8) or in synthetic minimal YC (0.12% yeast nitrogen base without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 0.1% succinic acid, 0.6% NaOH, 2% glucose and, as required, 2% agar) medium containing 0.075% amino acid supplements (lacking Trp, Leu, Ura, Lys, and His; 0.1% each of adenine sulphate, arginine, cysteine, threonine; 0.05% each of aspartic acid, isoleucine, methionine, phenylalanine, proline, serine, and tyrosine) buffered at pH5.8. When necessary 0.01% each of tryptophan, uracil, lysine, leucine and 0.005% histidine are supplemented to the media.

The *S. cerevisiae* reporter strain L40 (Stratagene) is employed. The genotype of the L40 yeast strain is MATa his3Æ200 trp1-901 leu2-3, 112 ade2 LYS::(lexAop)4-HIS URA3::(lexAop)8-LacZ GAL4 (Hollenberg, *et al.*, *Molecular and Cellular Biology* **15**: 3813-3822). Yeast cells are grown at 30°C or 20°C, as appropriate, for 72-144h.

Plasmids are transformed into L40 yeast strain using the lithium acetate transformation protocol (Gietz, *et al.* (1992) *Nucleic Acids Res* **20**: 1425). Positive clones are selected using auxotrophic markers for both plasmids and for lysine and histidine prototrophy. Histidine-positive colonies and controls are lysed in liquid nitrogen and assayed for β-galactosidase activity on filters as described (Breeden, L. & Nasmyth, K. (1985) *Cold Spring Harb Symp Quant Biol* **50**: 643-50).

LexA fusion baits are prepared in the plasmid pBTM116 (5.5 kb) (Invitrogen) (Bartel, *et al.*, (1993) in *Cellular interaction in development: a practical approach* (eds. Hartley, D.A.) 153-179, IRL Press, Oxford) contains the LexA domain, the Trp1 gene and the 2μm origin of replication. The AMCV p41 gene is amplified by RT-PCR from Artichoke Mottle Crinkle Virus cDNA (AMCV is kindly provided by Eugenio Benvenuto, ENEA, Dipartimento Innovazione, Divisione Biotecnologie e Agricoltura, Roma) using the primers:

5' - GCCCGAATTCATGGCAATGGTAAAGAGAAATAAT- 3' (sense); SEQ. ID. No. 1

5' - TTACAGGATCCCTAAATTAAAGAGACATCGTTGT- 3' (antisense); SEQ. ID. No. 2.

The PCR product is digested with EcoRI-BamHI, and inserted into EcoRI-BamHI sites of pBTM116.

ScFv-VP16 fusion clones are constructed in the plasmid pVP16* (7.5 kb) (Invitrogen). This vector is a modification of pVP16 that carries the LEU2 gene, a 2μm replication origin, the β-lactamase gene, two SV40 large antigen nuclear localisation sequences (NLS) fused to the VP16 acidic activation domain, with a upstream polylinker for cloning genes of interest to generate in-frame protein fusions to VP16, expressed from an ADH1 promoter (Vojtek, *et al.*, (1993) *Cell* **74**: 205-14). The scFvF8 gene (Tavladoraki, P., *et al.* (1993) *Nature* **366**: 469-472) is amplified by PCR from pGEMscFv(F8) (provided by Eugenio Benvenuto, ENEA, Dipartimento Innovazione, Divisione Biotecnologie e Agricoltura, Roma), a scFv made from a mouse monoclonal antibody raised against AMCV plant virus coat protein. The Mab is converted to scFv format and cloned into the pGEM expression prior to amplification using the primers:

5' -AATGGACTATGGCCCAGCCGGCCAATGCAGGTGCAGCTGCAGGAG- 3' (sense); SEQ. ID. No. 3;

5' -TCACCTGATAGCGGCCGCATTCAGATCCTCTTCTGAGAT- 3' (antisense); SEQ. ID. No. 4;

digested with SfiI-NotI, and inserted into SfiI-NotI sites of pVP16*.

If there is association of the two fusion proteins in yeast strain L40, transcription of two integrated reporter genes would occur. One is *HIS3* gene, which provides a nutritional selection for the two hybrid association and the other is the *lacZ* gene allowing a blue-white selection when the cells are grown on Xgal substrate. In this system, two nuclear localisation signals are located on the scFv-VP16 fusion vector, while the bait has none. Therefore, the interaction between the antigen (bait) and the scFv fragments (scFv-VP16

fusion) must occur in the cytoplasm, before the complex is translocated to the nucleus and activates transcription.

Co-expression of the Gal4-AMCV bait (AMCVp41/BTM116) and the scFvF8-VP16 fusion protein in yeast L40 cells leads to an efficient growth of the cells in the absence of histidine due to HIS3 activation and shows a high level of ß-galactosidase activity (Fig. 1, row 4). The co-transfection of the bait alone, the bait with the VP16 vector or with an unrelated antigen lamin-LexA fusion clone (Figure 1, rows 1, 2 and 3 respectively) does not result in activation of the *lacZ* gene. These results demonstrate that the scFvF8 is able to specifically interact with its corresponding antigen p41 under the intracellular conditions of this assay.

Example 2

Identification of Intracellular Binders

The use of yeast cells to detect intracellular antigen-scFv interaction further assessed with scFv derived either from monoclonal antibodies or from phage display antibody libraries, some of which have been shown to have intracellular activity when assayed *in vivo*. The binding of scFvs specific for HIV-1 integrase, the tyrosine kinase Syk and p21-ras is tested.

The anti-HIV integrase scFv IN33 is derived from a monoclonal antibody, and is expressed in human cells, leading to a specific neutralisation of HIV-1 integrase activity (Levy-Mintz, P., *et al.* (1996) *J. Virol.* **70**: 8821-8832). We have tested this scFv fused to VP16 in the yeast system (Figure 2A). ScFvIN33 (kindly provided by R.. Pomerantz, Jefferson Medical College, Dept. of Medicine, Philadelphia, USA) is amplified by PCR from pNLVP16 (Sadowski *et al.*, (1992) Gene 118:137-141), using the primers:

5' - AAAAAGAGAAAAGTGGCCCAGCCGGCCATGGGAATGGACATCCAGATGACA - 3' (sense); SEQ. ID. No. 5;

5' - GGCGGAGCTCGAGGCGGCCGCTGAGGAGACGGTGAGGCT- 3' (antisense); SEQ. ID. No. 6;

digested SfiI-NotI, and inserted into SfiI-NotI sites of VP16*.

HIV-1 integrase bait is constructed as follows: the HIV-1 IN33 gene is amplified by PCR from pRP1012 [6] by using the primers:

5' - GCTAGCCCGGGGATCCCAATGTTTCTAGATGGAATCGAT- 3' (sense); SEQ. ID. No.7;

5' - AGCCCCGGGATCCTGCAGCTAATCCTCATCCTGTCTACT - 3' (antisense); SEQ. ID. No. 8;

digested with BamHI-PstI, and inserted into BamHI-PstI sites of pBTM116.

When the HIV-1 IN/BTM116 bait is expressed with the scFv-VP16 fusion (scFvIN33/VP16) in L40 yeast, a high level of β-galactosidase activity is observed (Fig. 3A, row 4), whereas co-expression of the scFv-VP16 fusion with a lamin bait (row 3) or expression of the bait with alone or with the VP16 vector (rows 1 and 2 respectively) does not result in any activation These results establish the specificity and efficacy of the HIV Ag-scFv interaction in yeast cells.

The anti-HIV scFv IN33 is derived from a monoclonal antibody. In order to determine if phage scFv selected in vitro can function in the antibody-antigen two-hybrid assay. ScFv isolated from a phage display library with Syk protein (a non-receptor tyrosine kinase involved in signal transduction in B cells) are therefore tested for their ability to function in an intracellular environment. Three different scFv fragments, which specifically react with Syk in ELISA, immunoprecipitation, immunofluorescence and Western blots are fused to VP16 activation domain (scFvV6C11/VP16, scFvG4G11/VP16 and scFvG6G2/VP16) and tested in the yeast system (Fig. 2B). The plasmids pscFvV6C11-VP16*, pscFvG6G2-VP16 and pscFvG4G11-VP16 are constructed by subcloning NcoI-NotI fragments from pscFvexpV6C11, pscFvexpG6G2 and pscFvexpG4G11 (kindly

provided by Piona Dariavach, Institut de Genetique Moleculaire de Montpellier; an scFv isolated by phage display using Syk protein as antigen and subcloned into an expression vector) respectively into NcoI-NotI cut pCANTAB6 (McCafferty *et al.*, (1994) Applied Biochem. & Biotechnol. 47:160-171) and subcloning again SfiI-NotI fragments from pCANTAB6scFvV6C11, pCANTAB6scFvG6G2 and pCANTAB6scFvG4G11 respectively into SfiI-NotI cut VP16*.

In order to construct the Syk target (pLexA-Syk), the Syk gene is amplified by PCR from Syk cDNA (Fluck, *et al.*, (1995) *Biochem Biophys Res Commun* **213**: 273-81) by using the primers:

5' - GCCCGAATTCATGGCGGGAAGTGCTGTGGACAGCGCC -3' (sense); SEQ. ID. No. 9;

5' - TTACAGGATCCTTAGTTAACCACGTCGTAGTAGTAATTGCG -3' (antisense); SEQ. ID. No. 10;

digested with EcoRI-BamHI, and inserted into EcoRI-BamHI sites of pBTM116.

Only one of the scFv constructs, scFvG4G11, shows a positive interaction with the LexA-Syk bait (Syk/BTM116) as judged by the activation of β-galactosidase (Fig 2B, row 8) while the other two failed to interact (Figure 3B, rows 6 and 7). The specificity of scFvG4G11 interaction with the syk/BTM116 bait is established with a Lamin (Stratagene) bait vector (Fig. 2B, row 5). These data extend the notion that *in vitro* selection of scFv from phage display libraries is an inadequate criterion for their subsequent use as interacting intracellular antibodies. This inference that scFv selected purely for their ability to bind antigen *in vitro* (e.g. in Western blot assays) may not necessarily function correctly as intracellular antibodies is endorsed by finding that none of two anti-BCR, two anti-ABL or eight anti-MLL scFv selected from phage display libraries could elicit HIS3 or β-galactosidase activity.

All bait clones used in these assays are individually assessed for auto-activation of the *HIS3* gene with and without the empty pVP16 vector (Fig. 3A & 3B) and each is tested

by co-transfection with another bait encoding a lamin antigen and growth is detected in his$^-$ medium.

The conclusion that individual antibodies may not possess binding properties *in vivo* are re-enforced by findings obtained with a panel of scFv directed against the signal transduction protein p21 ras. There is a significant inhibition of signal transduction processes involving the activation of p21-ras when a anti-ras Y13-259 antibody is expressed in cells, either as a whole immunoglobulin (Biocca, S, *et al.*, (1994) *Biotechnology (N Y)* **12**: 396-9) or as scFv (Biocca, S., *et al.*, (1993) *Biochem Biophys Res Commun* **197**: 422-7; Cochet, O., *et al.* (1998) *Cancer Res* **58**: 1170-6; Cardinale, *et al.*, (1998) *FEBS Letters* **439,** 197-202). The scFv fragment Y13-259 is fused to the VP16 activation domain (scFvY13/VP16) and expressed in yeast cells, together with the K-ras protein fused to LexA (K-ras/BTM116), as follows:

For the scFv, pscFvY13-VP16* is constructed by subcloning SfiI-NotI fragments from pHENY13 (Hoogenboom *et al.*, (1991) Nucl. Ac. Res. 19:4133-7) into SfI-NotI cut pVP16*.

For the target, pLexA-K-Ras is constructed by amplifying the K-Ras B gene by PCR from pGem3Z-k-ras (a k-ras clone in pGEM kindly provided by Prof. Giancarlo Vecchio, Universita' degli Studi di Napoli FedericoII) using the primers:

5' - GATCGGATCCGTATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGT - 3' (sense); SEQ. ID. No. 11;

5' - GATCCTGCAGTTACATAATTACACACTTTG - 3' (antisense); SEQ. ID. No. 12;

digested with BamHI-PstI and cloned into BamHI-PstI sites of pBTM116.

The scFv fails to show β-galactosidase activity when the cells are grown at 30°C (Figure 2C, row 4). This is probably because this scFv aggregates intracellularly at higher

temperatures (Cardinale, *et al.*, (1998) *FEBS Letters* **439,** 197-202). On the other hand, the same co-transformed cells are able to grow in the absence of histidine and show an intense β-galactosidase activity if cultured at lower temperatures (20°C) (Figure 2C, row 5). This interaction is specific for the K-ras antigen (Figure 2C, row 3) and the K-ras bait does not activate the reporter genes on its own, at 20°C (Fig 2C, row 1 and 2) or 30°C. A number of other scFv fragments against the p21-ras protein, isolated from two different phage display libraries (Persic, L., *et al.* (1999) *FEBS Letters* 443:112-116) are also tested in this system. The binding affinity of each of these scFv fragments for p21-ras is determined by surface plasmon resonance, and shown to be in the range of 5-800 nM. However, only two out of twelve distinct scFv show any effect on the K-ras antigen, even when cells are grown at 20°C. Thus, not all scFv fragments isolated from a phage display library are able to bind their antigen when intracellularly expressed in the two hybrid system, not even those with high binding affinities for their antigen.

Example 3

Mammalian Intracellular Assay

Experiments with the yeast transfection assays indicate that antibody-antigen interaction can assemble the co-operative transcription complex necessary to transcribe the *HIS3* or *lacZ* genes, providing the means to determine if a particular antibody has the potential for use *in vivo* as an intracellular antibody. In practise, *in vivo* use of antibody fragments is mainly in higher organisms, such as in functional genomics or therapeutic uses for human diseases. Therefore the antibody-antigen two-hybrid assay system is evaluated in mammalian cells.

For the mammalian assay, pM-βgal is constructed by subcloning the 3.5kb β-galactosidase SfiI-PacI fragment of pN3neo2TK-1 (Dear, T.N., *et al.* (1995) *Development* **121**: 2909-2915) into the pM1 vector (Sadowski, *et al.* (1992) *Gene* **118**: 137-141) digested with SmaI. The ScFvR4 DNA fragment is amplified from pPM163R4 (Martineau, *et al.* (1998) *J Mol Biol* **280**: 117-127) by PCR using pfu DNA polymerase (Stratagene) and subcloned into pNLVP16 (EcoRI digested) or pNLVP16 (NdeI digested) to create pNL-ScFvR4-VP16 and pNLVP16-ScFvR4 in which the scFv segment is

respectively either N- or C-terminal to the VP16 element. pM-scFvR4 and pNLVP16-βgal are constructed by subcloning the ScFvR4 PCR product and βgal SfiI-PacI fragment into the pM1 and pNLVP16 vectors respectively. The EcoRI-BamHI AMCVp41 DNA fragment from AMCVp41/BTM116 is subcloned into pM1(EcoRI digested) to give pM1-AMCV. The ScFvF8 DNA is amplified from pGEM-ScFvF8 by PCR and cloned into pNLVP16 (EcoRI digested) to create pNLScFvF8-VP16. All clones are sequenced to confirm in-frame fusion of the inserts with the Gal4 binding domain or the VP16 in the vectors.

The Chinese hamster ovary (CHO) cell line is grown in α minimal essential medium (GIBCO BRL) with 10% foetal calf serum, penicillin and streptomycin. Transfection of CHO cells growing on 10mm diameter dishes at 50% confluence is performed using Lipofectin® reagent according to manufacturer's instructions (GIBCO BRL). 5μg of each plasmid is used in each transfection together with 5μg of pE5C-CAT reporter. pBSpt DNA is added where necessary to compensate for unequal DNA input in each transfection. Cells are harvested 48 hours after transfection and chloramphenicol acetyl-transferase (CAT) assay is performed as described previously (Masson, *et al.* (1998) *Mol. Cell. Biol.* **18**: 3502-3508). Each transfection is repeated twice and a PhosphorImager (Molecular Dynamics) is used to quantitate the intensity of the signals in the CAT assay.

Antigen fusions are made with the Gal4 DNA-binding domain within the vector pM1 and scFv fusions with pNLVP16 (Sadowski, *et al.* (1992) *Gene* **118**: 137-141) and co-transfections are performed in Chinese Hamster Ovary (CHO) cells with a chloramphenicol acetyl transferase (CAT) reporter vector. A panel of scFv which bind to AMCV, HIV-1 integrase, K-ras and β-galactosidase is tested. By contrast with the results obtained in yeast, only the latter activate the CAT reporter when co-expressed with the appropriate bait (the latter could not be used in yeast as it encodes β-galactosidase and is also found to auto-activate the *HIS3* gene promoter, Fig 4).

When CHO cells are co-transfected with the CAT reporter together with the DNA-binding pM-βgal bait and the pNLS-scFvR4-VP16 expression clones (encoding a fusion between the anti-β-galactosidase scFvR4 (Martineau, *et al.* (1998) *J Mol Biol* **280**: 117-

127) and the VP16 transcriptional transactivation domain), about 60 fold increase in CAT activity is detected (Figure 5A, lane 1) compared with the CAT reporter (lane 2). This indicates that the interaction of scFv with target antigen (i.e. β-galactosidase) in the CHO cells is sufficient to activate CAT transcription. The specificity of intracellular interaction of scFv with the β-galactosidase is determined by a number of controls. CHO cells are transfected with pM-βgal together with an non-relevant scFv-VP16 fusion construct (pNLscFvF8-VP16, Figure 4A, lane 5), or pNLscFvR4-VP16 together with a heterologous GAL4 binding domain-antigen fusion construct (pM1-AMCV, lane 6). Neither combination activates the CAT reporter indicating that the intracellular interaction between β-gal and scFvR4 is a specific one. In addition, the fusion of the scFvR4 with the GAL4 DNA-binding domain and of β-galactosidase with the VP16 activation domain retains the ability to activate the CAT reporter, albeit with about 50% lower level (Fig. 4A lane 7: duplicate experiments are analysed and the data presented as a histogram, Figure 4B, lane 7). The lower efficiency may due to structural constraints as a construct expressing an scFvR4 linked at the C-terminus of the VP16 activation domain also showed reduced CAT activation (Fig. 4A and 4B, lanes 10). Therefore a plausible explanation is that the VP16 at the amino-terminus in this fusion caused a stearic hindrance to the antigen binding of the scFv.

Despite the efficacy of antibody fragments in a range of functional tests, the inability of many of our scFv panel to interact with antigen intracellularly (in yeast or CHO cells) presumably reflects the relatively low affinity or structural stability of those scFv in cells. The anti-AMCV scFvF8 is able to fold sufficiently well to bind antigen in plant cells (Tavladoraki, P., *et al.* (1993) *Nature* **366**: 469-472) and in the interaction shown here. The redox state of the scFvF8 expressed as the VP16 fusion protein in yeast cells is therefore investigated. Protein is extracted from yeast transfected with scFvF8-VP16 and fractionated on SDS-PAGE with or without reduction by β-mercaptoethanol (Figure 5). No difference is seen in the mobility of the scFvF8 protein when reduced or unreduced, as compared to a clear difference in electrophoretic mobility between the oxidised and the reduced forms of a secretory scFv fragment made in baculovirus and which has disulphide bonds formed (Fig. 5). Thus it appears that scFvF8 made in yeast does not have VH-VL intrachain S-S bonds and is intrinsically stable in the absence of disulphide

bonds. The corollary is that the yeast antibody-antigen hybrid assay should be capable of selecting those antibody fragments that can tolerate the absence of the intrachain disulphide bond, and retain antigen binding.

Example 4

Selection of scFv in the yeast antibody-antigen system

To verify the applicability of the method of the invention to selection of intracellular antibodies from a library of antibody molecules, a selection is carried out using the AMCVp41/BTM116 bait with scFvF8-VP16 fusion progressively diluted with DNA from a library encoding various non-relevant scFv-VP16 fusion proteins (Figure 6) at ratios of 1:1, 1:10 and 1:100 scFvF8-VP16: scFv library . The DNA mixtures are co-transformed into yeast cells, which are grown in the absence or in the presence of histidine (Figure 6, top plates: YC-WHULK and bottom plates YC-WL, respectively) for selection. After 3 days, colonies are picked and replated on a grid plate (with or without histidine as appropriate) and these colonies are screened for β-galactosidase activity. When the yeast are grown in the absence of histidine, only those with both AMCVp41/BTM116 and scFvF8-VP16 protein expression are able to grow and all the surviving colonies are capable of activation of the β-gal gene due to interaction of p41 and scFv (Fig. 6, top row). However, in the absence of selection (i.e. HIS^+ growth) all yeast can grow since under these conditions cells are selected only for the presence of scFv-VP16 fusion and bait plasmids. Nonetheless, we are able to detect the scFvF8-VP16-bait interaction from transfections at dilutions up to 100 times (Fig. 6, bottom row). Selection strategies at much higher dilutions are thus clearly feasible.

Claims

1. A method for determining the ability of an antibody to bind to an antigen in an intracellular environment, comprising the steps of:

a) providing a first molecule and a second molecule, wherein stable interaction of the first and second molecules leads to the generation of a signal;

b) providing an intracellular antibody which is associated with the first molecule;

c) providing an intracellular antigen which is associated with the second molecule, such that association of the antibody and the antigen leads to stable interaction of the first and second molecules and generation of the signal;

d) assessing the intracellular interaction between the antibody and the antigen by monitoring the signal.

2. A method according to claim 1, wherein the first and/or second molecules are polypeptides.

3. A method according to claim 2, wherein the first and second molecules associate to form an active reporter molecule.

4. A method according to claim 3, wherein the active reporter molecule is selected from the group consisting of a transcription factor, an enzyme and a bioluminescent molecule.

5. A method according to claim 4 wherein the active reporter molecule is an enzyme and the method is performed in the presence of a substrate for the enzyme.

6. A method according to any one of claims 3 to 5, wherein the first and second molecules are domains of the active reporter molecule.

7. A method according to claim 6, wherein the first molecule is the activation domain of VP16 and the second molecule is the DNA-binding domain of LexA.

8. A method according to any preceding claim, wherein the signal is selected from the group consisting of a change in an optical property and the activation of a reporter gene.

9. A method according to claim 8, wherein the signal allows the sorting of cells.

10. A method according to any preceding claim wherein the antibody is selected from the group consisting of an intact immunoglobulin, a Fv, a scFv, a Fab and a $F(ab')_2$.

11. A method according to any preceding claim, wherein the antibody is provided by expressing an antibody-encoding nucleic acid within the cell.

12. A method according to claim 11, wherein the antibody-encoding nucleic acid is obtained from a library of antibody-encoding nucleic acids.

13. A method according to claim 12, wherein the library is a phage library encoding a repertoire of antibodies.

14. A method according to claim 12, wherein the library is constructed from nucleic acids isolated from an organism which has been challenged with an antigen.

15. A method according to claim 1, comprising the further step of:
e) isolating those antibodies which give rise to a signal.

16. A method according to claim 15, comprising the further step of
f) subjecting the selected antibodies to a functional intracellular assay.

17. A method according to any preceding claim, wherein one or both of the antibody and the antigen, together with the first or second molecules, are provided in the form of nucleic acid constructs which are transcribed to produce said antibody and/or antigen together with said first or second molecules.

18. A method for preparing an antibody suitable for use in a procedure according to claim 1, comprising the steps of:

a) expressing a repertoire of antibody genes in a selection system and isolating those genes which encode antibodies specific for a desired antigen;

b) bringing the isolated genes into operative association with nucleic acids encoding a first molecule, wherein stable interaction of the first molecule with a second molecule generates a signal, in order to produce a fusion polypeptide comprising the antibody and the first molecule.

Amendments to the claims have been filed as follows

Claims

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1. A method for determining the ability of an immunoglobulin to bind to a target in an intracellular environment, comprising the steps of:

a) providing a first molecule and a second molecule, wherein stable interaction of the first and second molecules leads to the generation of a signal;

b) providing an intracellular immunoglobulin which is associated with the first molecule;

c) providing an intracellular target which is associated with the second molecule, such that association of the immunoglobulin and the target leads to stable interaction of the first and second molecules and generation of the signal;

d) assessing the intracellular interaction between the immunoglobulin and the target by monitoring the signal.

2. A method according to claim 1, wherein the first and/or second molecules are polypeptides.

3. A method according to claim 2, wherein the first and second molecules associate to form an active reporter molecule.

4. A method according to claim 3, wherein the active reporter molecule is selected from the group consisting of a transcription factor, an enzyme and a bioluminescent molecule.

5. A method according to claim 4 wherein the active reporter molecule is an enzyme and the method is performed in the presence of a substrate for the enzyme.

6. A method according to any one of claims 3 to 5, wherein the first and second molecules are domains of the active reporter molecule.

7. A method according to claim 6, wherein the first molecule is the activation domain of VP16 and the second molecule is the DNA-binding domain of LexA.

8. A method according to any preceding claim, wherein the signal is selected from the group consisting of a change in an optical property and the activation of a reporter gene.

9. A method according to claim 8, wherein the signal allows the sorting of cells.

10. A method according to any preceding claim wherein the immunoglobulin is selected from the group consisting of an intact immunoglobulin, a Fv, a scFv, a Fab and a $F(ab')_2$.

11. A method according to any preceding claim, wherein the immunoglobulin is provided by expressing an immunoglobulin-encoding nucleic acid within the cell.

12. A method according to claim 11, wherein the immunoglobulin-encoding nucleic acid is obtained from a library of immunoglobulin-encoding nucleic acids.

13. A method according to claim 12, wherein the library is a phage library encoding a repertoire of immunoglobulins.

14. A method according to claim 12, wherein the library is constructed from nucleic acids isolated from an organism which has been challenged with an antigen.

15. A method according to claim 1, comprising the further step of:
e) isolating those immunoglobulins which give rise to a signal.

16. A method according to claim 15, comprising the further step of
f) subjecting the selected immunoglobulins to a functional intracellular assay.

17. A method according to any preceding claim, wherein one or both of the immunoglobulin and the target, together with the first or second molecules, are provided in the form of nucleic acid constructs which are transcribed to produce said immunoglobulin and/or target together with said first or second molecules.

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Application No: GB 9905510.5
Claims searched: 1-17

Examiner: L.V.Thomas
Date of search: 12 August 1999

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.Q):

Int Cl (Ed.6):

Other: Online: EPODOC, WPI, BIOSIS, SCISEARCH

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
A	WO 96/40248 A1 (EUKARION) see p.5 l.16 - p.6 l.14, p.7 l.32 - p.8 l.37 and p.15 l.27 - p.16 l.17	1
A	US 4499183 (SUJANSKY ET AL.) see col.3 ll.18-59	1
A	J. Clin. Lab. Anal. 1987 1(1) pp.56-61 - Ahearn et al. - see Introduction and abstract	1

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
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